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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In the Application of:

Lawton *et al.*

Serial No.: 10/054,647

Filed: January 22, 2002

For: Compositions and Methods for Detection
of *Ehrlichia canis* and *Ehrlichia*
chaffeensis Antibodies

Art Unit: 1645

Examiner: Ford, V.

Conf. No. 9151

Atty. Docket No.: 00-1278-C

BRIEF ON APPEAL

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of <i>Ehrlichia canis</i> and <i>Ehrlichia</i>)	Atty. Docket No.: 00-1278-C
<i>chaffeensis</i> Antibodies)	

BRIEF ON APPEAL

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

This appeal brief is submitted along with the large entity fee of three hundred forty dollars (\$340). In the event of any variance between any of the amounts enclosed and the Patent and Trademark Office charges, the Commissioner is authorized to charge or credit any difference to our Deposit Account No. 13-2490.

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REAL PARTY IN INTEREST

The real party in interest is IDEXX Laboratories, Inc., Westbrook, Maine, to whom this invention is assigned.

RELATED APPEALS AND INTERFERENCES

A related appeal brief has been filed on July 23, 2004, in U.S. Serial No. 09/765,739, of which this application is a divisional. An appeal brief has also been filed on October 21, 2004, in U.S. Serial No. 10/054,354, which is also a divisional application of U.S. Serial No. 09/765,739. No decisions by a court or the Board have been issued in these related applications. Applicant is aware of no other related appeals, interferences, or judicial proceedings concerning this application.

STATUS OF CLAIMS

Claims 1-13 are pending and stand rejected. Claims 1-13 are being appealed. A copy of the claims is attached in Appendix A.

STATUS OF AMENDMENTS

No amendments were presented after final.

RESERVATION OF RIGHT TO SUPPLEMENT APPEAL BRIEF

A Final Office Action was issued on May 19, 2004. A response to the Office Action was timely filed on July 13, 2004. A notice of appeal was filed on August 19, 2004. No advisory action has been issued in this application. Applicants respectfully reserve the right to supplement this brief in response to any issued office action.

SUMMARY OF THE INVENTION

The invention provides a composition of matter comprising an isolated polypeptide consisting essentially of SEQ ID NO:2 or a phenotypically silent amino acid substitution variant

thereof that specifically binds to an anti-*Ehrlichia* antibody. See Table 1; page 7, lines 14 through page 8, line 20; page 9, lines 8-11. The composition can further comprise a carrier. See page 11, lines 10-15.

The invention also provides an article of manufacture comprising packaging material and, contained within the packaging material, a polypeptide comprising the polypeptide consisting essentially of SEQ ID NO:2 or a phenotypically silent amino acid substitution variant thereof that specifically binds to an anti-*Ehrlichia* antibody. See page 4, lines 6-12; page 15, line 22 through page 16, line 1; Table 1; page 7, lines 14 through page 8, line 20; page 9, lines 8-11; page 4, lines 10-12. The packaging material can comprise a label that indicates that the polypeptide can be used for the identification of *Ehrlichia* infection in a mammal. See page 4, lines 6-12; page 15, line 22 through page 16, line 1; Table 1; page 7, lines 14 through page 8, line 20; page 9, lines 8-11; page 4, lines 10-12. The label can indicate that identification of an *Ehrlichia* infection is done using a method of detecting presence of antibodies to *Ehrlichia* comprising:

(a) contacting a polypeptide the polypeptide consisting essentially of SEQ ID NO:2 or a phenotypically silent amino acid substitution variant of the polypeptide consisting essentially of SEQ ID NO:2 that specifically binds to an anti-*Ehrlichia* antibody, with a test sample suspected of comprising antibodies to *Ehrlichia*, under conditions that allow polypeptide/antibody complexes to form; and

(b) detecting polypeptide/antibody complexes;

wherein the detection of polypeptide/antibody complexes is an indication that an *Ehrlichia* infection is present. See page 4, lines 13-21. The *Ehrlichia* infection can be caused by *Ehrlichia canis* or *Ehrlichia chaffeensis*. See page 11, line 22 through page 12, line 1.

The invention also provides a composition of matter comprising an isolated polypeptide consisting essentially of SEQ ID NO:2 or a conservative amino acid substitution variant thereof that specifically binds to an anti-*Ehrlichia* antibody. See Table 1; page 6, lines 3-12; page 7, lines 14 through page 8, line 20; page 9, lines 8-11.

Furthermore, the invention provides an article of manufacture comprising packaging material and, contained within the packaging material, a polypeptide comprising the polypeptide consisting essentially of SEQ ID NO:2 or a conservative amino acid substitution variant thereof that specifically binds to an anti-*Ehrlichia* antibody. See page 4, lines 6-12; page 15, line 22 through page 16, line 1; Table 1; page 6, lines 3-12; page 7, lines 14 through page 8, line 20; page 9, lines 8-11; page 4, lines 10-12. The label can indicate that identification of an *Ehrlichia* infection is done using a method of detecting presence of antibodies to *Ehrlichia* comprising:

(a) contacting a polypeptide comprising the polypeptide consisting essentially of SEQ ID NO:2 or a conservative amino acid substitution variant of the polypeptide consisting essentially of SEQ ID NO:2 that specifically binds to an anti-*Ehrlichia* antibody, with a test sample suspected of comprising antibodies to *Ehrlichia*, under conditions that allow polypeptide/antibody complexes to form; and

(b) detecting polypeptide/antibody complexes;

wherein the detection of polypeptide/antibody complexes is an indication that an *Ehrlichia* infection is present. See page 4, lines 13-21.

Additionally, the invention provides a composition of matter comprising an isolated polypeptide that is 20 amino acids in length, which comprises SEQ ID NO:2 or a phenotypically silent amino acid substitution variant thereof that specifically binds to an anti-*Ehrlichia* antibody.

See Table 1; page 7, lines 14 through page 8, line 20; page 9, lines 8-11. The composition can further comprise a carrier. See page 11, lines 10-15.

The invention also provides a composition of matter comprising an isolated polypeptide that is 20 amino acids in length, which comprises SEQ ID NO:2 or a conservative amino acid substitution variant thereof that specifically binds to an anti-*Ehrlichia* antibody. See Table 1; page 6, lines 3-12; page 7, lines 14 through page 8, line 20; page 9, lines 8-11. The composition can further comprise a carrier. See page 11, lines 10-15.

GROUND OF REJECTION TO BE REVIEWED ON APPEAL

- I. Claims 1-13 stand rejected under 35 U.S.C. §112, first paragraph as allegedly lacking written description.
- II. Claims 1-13 are stand rejected under 35 U.S.C. § 112, first paragraph as allegedly lacking enablement.
- III. Claims 1-13 stand rejected under 35 U.S.C. §102(a) as allegedly anticipated by Rikihisa *et al.* WO 99/13720 (“Rikihisa”).
- IV. Claims 1-9 stand rejected under 35 U.S.C. §112, second paragraph as allegedly indefinite.

ARGUMENT

I. **Claims 1-13 have adequate written description under 35 U.S.C. §112, first paragraph.**

Claims 1-13 stand rejected under 35 U.S.C. §112, first paragraph as allegedly lacking written description. Applicants respectfully traverse the rejection.

The Office Action asserts that “there is nothing on the record to show that the specification is enabled for the full scope of the claims and therefore does not meet the written description requirement.” *See* page 4, third full paragraph. The Office appears to confuse the standards for written description and enablement. The written description and enablement requirements of the first paragraph of 35 U.S.C. §112 are separate and distinct requirements and each have their own standard. *See e.g., Vas-Cath Inc. v. Mahurkar*, 19 USPQ2d 1111, 118 (Fed. Cir.1991).

The standard for written description requires that one of skill in the art must recognize that the applicant was in possession of the claimed genus, that is, variants of
SEQ ID NO:2. Importantly:

The written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species by actual reduction to practice, reduction to drawings, or by disclosure of relevant, identifying characteristics, i.e. structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics, sufficient to show the applicant was in possession of the claimed genus. Guidelines for Examination of Patent Applications Under the 35 U.S.C. 112, ¶1, “Written Description” Requirement, 66 Fed. Reg. 1099, 1106 (2001) (citations omitted).

Satisfactory disclosure of a representative number of species depends on whether one of skill in the art would recognize that the applicant was in possession of the necessary common attributes or features of the elements possessed by the members of the genus in view of the species disclosed. Description of a representative number of species does not require the description to be of such specificity that it would provide individual support for each species that the genus embraces. One species can adequately support a genus.

What is a representative number of species depends on whether one of skill in the art would recognize that the Applicants were in possession of the necessary common attributes or features of the elements possessed by the members of the genus in view of the species disclosed or claimed. Distinguishing characteristic include:

- A. partial structure;
- B. physical and/or chemical properties;
- C. functional characteristics;
- D. known or disclosed correlation between structure and function;
- E. method of making; and
- F. combinations of A-E.

All of these factors, in view of the level of skill and knowledge in the art in light of and consistent with the written description, should be considered. See M.P.E.P. § 2163.

In the instant case, the partial structure of claimed variants are known, *i.e.*, sequences that having at least 85% identity to SEQ ID NO:2. See specification, page 5, lines 8-11. Therefore, variants have about 17 amino acids in common with the 20 amino acid long SEQ ID NO:2. The specification also discloses 7 related sequences that are

useful in the invention, which were discovered using phage display technology. See specification, page 6. When the sequence of SEQ ID NO:2 is compared to the sequences of SEQ ID NOs:1, and 3-7, highly conserved amino acids and partially conserved amino acids are revealed.

Table 1.

SEQ ID NO	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	K	S	T	V	G	V	F	G	L	K	H	D	W	D	G	S	P	I	L	K
2	N	T	T	T	G	V	F	G	L	K	Q	D	W	D	G	A	T	I	K	D
3	N	T	T	V	G	V	F	G	L	K	Q	N	W	D	G	S	A	I	S	N
4	N	P	T	V	A	L	Y	G	L	K	Q	D	W	N	G	V	S	A		
5	N	T	T	V	G	V	F	G	L	E	Q	D	W	D	R	C	V	I	S	
6	N	P	T	V	A	L	Y	G	L	K	Q	D	W	E	G	I	S	S		
7	N	T	T	T	G	V	F	G	L	K	Q	D	W	D	G	S	T	I	S	

Table 1 demonstrates that positions 3, 8, and 13 of SEQ ID NO:2 are highly conserved across the seven sequences (dark gray columns). Additionally, positions 1, 4, 5, 6, 7, 9, 10, 11, 12, and 15 are partially conserved across the 7 sequences (light gray columns). That is, only 2 different amino acids appear in these positions. For instance, only K or N appear as amino acids in position 1 across the seven sequences. One of skill in the art would recognize that variants should likely retain the amino acids at positions 3, 8, and 13 and that one of two amino acids should likely be present at positions 1, 4, 5, 6, 7, 9, 10, 11, 12, and 15. One of skill in the art would also recognize that amino acids at position 2, 14, and 16-20 could tolerate a greater range of amino acid substitutions. Therefore, the specification provides structural guidance for the claimed variants.

Each of the claims recite that the isolated polypeptides specifically bind to an anti-*Ehrlichia* antibody. The physical properties and functional characteristics of the

variants are disclosed in the instant specification. That is, the specification teaches that the variants specifically bind to an anti-*Ehrlichia* antibody and also teaches how to test if such variants specifically bind to an anti-*Ehrlichia* antibody. *See* specification page 10, line 6 through page 11, line 6; page 11, line 21- page 16, line 8; Example 1. Methods of making the variants of SEQ ID NO:2 are well-known in the art and are described in the specification. *See e.g.* page 5, lines 7-14; page 6, line 3 through page 7, line 5; page 7, line 12 through page 9, line 7; page 18, line 19 through page 19, line 13; page 7, line 6 through page 9, line 7. One of skill in the art could make and test variants of invention given the specification and the knowledge in the art.

Therefore, one of skill in the art would recognize that applicant was in possession of the necessary common attributes of features of the elements possessed by the members of the genus in view of the species disclosed because the partial structure, physical and/or chemical properties, functional characteristics, and methods of making the claimed variants is disclosed in the specification. The written description does not have to be of such specificity that it would provide individual support for each species that the genus embraces.

Therefore, when all factors are considered, one of skill in the art would recognize from the disclosure that the Applicants were in possession of the claimed invention. Applicants respectfully request withdrawal of the rejection.

II. Claims 1-13 are enabled under 35 U.S.C. §112, first paragraph.

Claims 1-13 stand rejected under 35 U.S.C. § 112, first paragraph as allegedly lacking enablement. Applicants respectfully traverse the rejection.

The Final Office Action asserts that the claimed variants are not enabled by the specification because there is allegedly no description of the SEQ ID NO:2 mutational sites, the specification discloses only SEQ ID NO:2, and the specification fails to provide guidance as to which amino acids can be changed so that the variant polypeptides still retain their claimed biological function.

However, a structural description of the claimed variants is indeed provided by the specification. The variants are phenotypically silent or conservative amino acid substitution variants that have at least 85% identity to SEQ ID NO:2, and specifically bind to an anti-*Ehrlichia* antibody. See page 5, lines 8-11; page 9, lines 8-11. Since SEQ ID NO:2 is about 20 amino acids long, an amino acid substitution variant has only about 3 amino acid substitutions at the most. The specification also discloses 7 related sequences that are useful in the invention, which were discovered using phage display technology. See specification, page 6. When the sequence of SEQ ID NO:2 is compared to the sequences of SEQ ID NOs:1, and 3-7, highly conserved amino acids and partially conserved amino acids are revealed. See Table 1, *supra*.

Table 1 demonstrates that positions 3, 8, and 13 of SEQ ID NO:1 are highly conserved across the seven sequences (dark gray columns). Additionally, positions 1, 4, 5, 6, 7, 9, 10, 11, 12, and 15 are partially conserved across the 7 sequences (light gray columns). That is, only 2 different amino acids appear in these positions. For instance, only K or N appear as amino acids in position 1 across the seven sequences. One of skill in the art would recognize that the claimed variants should likely retain the amino acids at positions 3, 8, and 13 and that one of two amino acids should likely be present at positions 1, 4, 5, 6, 7, 9, 10, 11, 12, and 15. One of skill in the art would also recognize

that amino acids at positions 2, 14, and 16-20 could tolerate a greater range of amino acid substitutions. Therefore, the specification provides structural guidance as to which amino acids can be changed so that the variant polypeptides retain their biological function.

The standard for enablement is whether one reasonably skilled in the art (1) could make and use the invention (2) from the disclosures in the patent coupled with information known in the art (3) without undue experimentation. As taught in the specification and described above, one of skill in the art could make the claimed variants while maintaining binding to an anti-*Ehrlichia* antibody, without undue experimentation. See e.g., specification at page 5, line 6 through page 11, line 20. Therefore, the claims are enabled. Applicants respectfully request withdrawal of the rejection.

III. Claims 1-13 are novel over Rikihisa under 35 U.S.C. §102(a)

Claims 1-13 stand rejected under 35 U.S.C. §102(a) as allegedly anticipated by Rikihisa *et al.* Applicants respectfully traverse the rejection.

The Final Office Action asserts that Rikihisa teaches an antigen (*i.e.*, an isolated polypeptide) used to detect the presence of antibodies to *E. canis*. The Office Action argues that since the invention encompasses variants of SEQ ID NO:2, one of skill in the art could conclude that the *E. canis* polypeptides of Rikihisa are variants of SEQ ID NO:2.

The claims are drawn to compositions and articles of manufacture comprising an isolated polypeptide consisting essentially of SEQ ID NO:2 and conservative amino acid substitution variants and phenotypically silent amino acid variants thereof. The transitional phrase “consisting essentially of” limits the scope of a claim to the specified materials or steps “and those that do not materially affect the basic and novel

characteristic(s) of the claimed invention.” See, *In re Herz*, 190 USPQ 461, 463 (CCPA 1976) (emphasis in original); MPEP §2111.03.

The claims recite an isolated *E. canis* polypeptide, i.e., a portion or fragment of the whole protein. Rikihisa does not teach or suggest polypeptide portions or fragments. The isolated polypeptides of the instant invention are useful, *inter alia*, to detect the presence of anti-*Ehrlichia* antibodies. The claimed isolated polypeptides can be used as reagents in assays that provide greater sensitivity than the reagents taught in Rikihisa (*i.e.*, whole, recombinant proteins). See declaration of Dr. Chandrashekar, paragraphs 2-3 and 6-7 (of record; copy attached in Appendix B). The specification also teaches that:

currently known assays for detecting anti-*Ehrlichia* antibodies or fragments thereof are severely limited in usefulness because of sensitivity and specificity issues directly related to the impure nature of the *Ehrlichia* antigen used in these tests. See page 2, line 25 through page 3, line 1.

The Office appears to assert that the full-length proteins taught by Rikihisa would teach or suggest the claimed isolated polypeptides. Initially, Rikihisa does not teach or suggest the claimed isolated polypeptides and does not teach or suggest any portions or fragments of the full-length proteins that would specifically bind an anti-*Ehrlichia* antibody.

Furthermore, the addition of amino acids to the claimed isolated polypeptides so they encompass the whole proteins of Rikihisa would materially affect the basic and novel characteristics of the polypeptides. That is, use of full-length proteins would result in assays that are less sensitive than those disclosed in the instant specification. As such, the claims cannot be read so that the whole proteins of Rikihisa encompass the claimed isolated polypeptides.

Rikihisa does not anticipate claims 1-13 because Rikihisa does not teach, suggest, or inherently disclose each and every element of claims 1-13. Applicants respectfully request withdrawal of the rejection.

IV. Claims 1-9 are definite under 35 U.S.C. §112, second paragraph.

Claims 1-9 stand rejected under 35 U.S.C. §112, second paragraph as allegedly lacking definiteness. Applicants respectfully traverse the rejection.

The Office Action asserts that claims 1-9 are indefinite for the use of the term “phenotypically silent amino acid substitution variants.” Initially, claims 7, 8 and 9 do not contain this term. As such, the rejection is improper for these claims. The Office Action asserts that the metes and bounds of “phenotypically silent amino acid substitutions” cannot be ascertained by what is disclosed in the specification. However, the test for definiteness is whether “those skilled in the art would understand what is claimed when the claim is read in light of the specification.” *Orthokinetics, Inc. v. Safety Travel Chairs, Inc.*, 1 USPQ2d 1081, 1088 (Fed. Cir.1986).

One of skill in the art would understand the meaning of phenotypically silent amino acid substitution variants given the specification. The specification teaches phenotypically silent amino acid substitution variants and how to identify such variants at, *inter alia*, page 7, line 10 through page 9, line 7. In particular, the specification teaches that a phenotypically silent amino acid substitution is an amino acid substitution that has little effect on activity of the variant polypeptide. One of skill in the art would understand, in light of the specification and the state of the art, that variants of SEQ ID NO:2 that have about the same biological activity of a polypeptide of SEQ ID NO:2 are encompassed by “phenotypically silent amino acid variants”. Additionally, the claims

recite that a phenotypically silent amino acid variant specifically binds an anti-*Ehrlichia* antibody. One of skill in the art would further understand what is claimed because the claim provides functional language for the variants, which is described in detail in the specification.

The claims are therefore definite and applicants respectfully request withdrawal of the rejection.

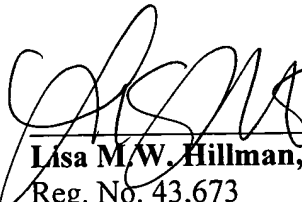
Summary

Applicants respectfully submit that the claims are in a condition for allowance.

Respectfully submitted,

Date: OCT 21, 2004

by:



Lisa M.W. Hillman, PhD
Reg. No. 43,673



PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
(Case No. 00-1278-C)

In the Application of:
Lawton, et al.

Serial No.: 10/054,647

Filed: January 22, 2002

For: Compositions and Methods for Detection
Of *Ehrlichia canis* and *Ehrlichia*
chaffeensis Antibodies

Art Unit: 1645

Examiner: Ford, V.

Conf. No. 9151

TRANSMITTAL LETTER

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

In regard to the above identified application,

1. We are transmitting herewith the attached:
 - a) Appeal Brief;
 - b) Appendices A and B;
 - c) Filing fee; and
 - d) Return postcard
2. With respect to fees:
 - a) A check in the amount of \$340.00 is enclosed.
 - b) Please charge any underpayment or credit any overpayment our Deposit Account, No. 13-2490.
3. GENERAL AUTHORIZATION: Please charge any additional fees or credit overpayment to Deposit Account No. 13-2490. A duplicate copy of this sheet is enclosed.



PATENT

CERTIFICATE OF MAILING UNDER 37 CFR § 1.8: The undersigned hereby certifies that this Transmittal Letter and the paper, as described in paragraph 1, are being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on October 21, 2004.

Date: October 21, 2004

Respectfully submitted,

A handwritten signature in dark ink, appearing to read "Lisa M.W. Hillman", written over a horizontal line.

Lisa M.W. Hillman

Registration No. 43,673

CLAIMS AS PENDING



APPENDIX A

1. (Previously Presented) A composition of matter comprising an isolated polypeptide consisting essentially of SEQ ID NO:2 or a phenotypically silent amino acid substitution variant thereof that specifically binds to an anti-*Ehrlichia* antibody.
2. (Previously Presented) The composition of claim 1, further comprising a carrier.
3. (Previously Presented) An article of manufacture comprising packaging material and, contained within the packaging material, a polypeptide comprising the polypeptide consisting essentially of SEQ ID NO:2 or a phenotypically silent amino acid substitution variant thereof that specifically binds to an anti-*Ehrlichia* antibody.
4. (Previously Presented) The article of manufacture of claim 3 wherein the packaging material comprises a label that indicates that the polypeptide can be used for the identification of *Ehrlichia* infection in a mammal.
5. (Previously Presented) The article of manufacture of claim 4, wherein the label indicates that identification of an *Ehrlichia* infection is done using a method of detecting presence of antibodies to *Ehrlichia* comprising:
 - (a) contacting a polypeptide the polypeptide consisting essentially of SEQ ID NO:2 or a phenotypically silent amino acid substitution variant of the polypeptide consisting essentially of SEQ ID NO:2 that specifically binds to an anti-*Ehrlichia* antibody, with a test sample suspected of comprising antibodies to *Ehrlichia*, under conditions that allow polypeptide/antibody complexes to form; and
 - (b) detecting polypeptide/antibody complexes;wherein the detection of polypeptide/antibody complexes is an indication that an *Ehrlichia* infection is present.

6. (Previously Presented) The article of manufacture of claim 4, wherein the *Ehrlichia* infection is caused by *Ehrlichia canis* or *Ehrlichia chaffeensis*.
7. (Previously Presented) A composition of matter comprising an isolated polypeptide consisting essentially of SEQ ID NO:2 or a conservative amino acid substitution variant thereof that specifically binds to an anti-*Ehrlichia* antibody.
8. (Previously Presented) An article of manufacture comprising packaging material and, contained within the packaging material, a polypeptide comprising the polypeptide consisting essentially of SEQ ID NO:2 or a conservative amino acid substitution variant thereof that specifically binds to an anti-*Ehrlichia* antibody.
9. (Previously Presented) The article of manufacture of claim 4, wherein the label indicates that identification of an *Ehrlichia* infection is done using a method of detecting presence of antibodies to *Ehrlichia* comprising:
- (a) contacting a polypeptide comprising the polypeptide consisting essentially of SEQ ID NO:2 or a conservative amino acid substitution variant of the polypeptide consisting essentially of SEQ ID NO:2 that specifically binds to an anti-*Ehrlichia* antibody, with a test sample suspected of comprising antibodies to *Ehrlichia*, under conditions that allow polypeptide/antibody complexes to form; and
 - (b) detecting polypeptide/antibody complexes;
- wherein the detection of polypeptide/antibody complexes is an indication that an *Ehrlichia* infection is present.
10. (Previously Presented) A composition of matter comprising an isolated polypeptide that is 20 amino acids in length, which comprises SEQ ID NO:2 or a phenotypically silent amino acid substitution variant thereof that specifically binds to an anti-*Ehrlichia* antibody.
11. (Previously Presented) The composition of claim 10, further comprising a carrier.

12. (Previously Presented) A composition of matter comprising an isolated polypeptide that is 20 amino acids in length, which comprises SEQ ID NO:2 or a conservative amino acid substitution variant thereof that specifically binds to an anti-*Ehrlichia* antibody.

13. (Previously Presented) The composition of claim 12, further comprising a carrier.

APPENDIX B

A copy of a declaration signed by Dr. Chandrasheka and submitted under Rule 1.132 is attached. The declaration was entered into the record on February 23, 2004.



UNITED STATES PATENT AND TRADEMARK OFFICE
(Case No. 00-1278)

PATENT

In the Application of:

Lawton, et al.

Serial No.: 09/765,739

Filed: January 18, 2001

Art Unit: 1645

Examiner: V. Ford

For: Compositions and Methods for Detection of *Ehrlichia canis* and *Ehrlichia chaffeensis* Antibodies

DECLARATION UNDER 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

1. I, Ramaswamy Chandrashekar, am a research scientist for IDEXX Laboratories, Inc., the entire Assignee of U.S. Pat. Appl. Ser. Nos. 09/765,739, 10/054,354, and 10/054,647. I have earned a Ph.D. in Biochemistry and M.S. in Zoology. I have performed research and development in the field of sero-diagnostics for veterinary bacterial pathogens, including, for example, *Ehrlichia* ssp. and *Streptococcus equi* for over five years. In addition, I have performed research and development in the field of sero-diagnostics for nematode infections in both humans and animals for over 20 years. I am an author of over 70 scientific publications in the field of diagnosis and prevention of nematode infections. A copy of my Curriculum Vitae is attached.

2. Waner et al., (*J. Vet. Diagn. Invest.*, Vol. 12, pp. 240-244, 2000), Cadman et al., (*The Veterinary Record*, 135, 362), Rikihisa (WO 99/13720), and other references

that teach or suggest the use of entire *E. canis* or *E. chaffeensis* infected cells, whole (i.e., non-fragmented) *E. canis* or *E. chaffeensis* proteins, including mixtures of whole proteins, natural whole proteins, or whole recombinant proteins, to, e.g., detect *Ehrlichia*, do not teach or suggest polypeptides of SEQ ID NOs:1-7 to, e.g., detect *Ehrlichia*. As explained in the specifications of the above-mentioned patent applications, entire *E. canis* or *E. chaffeensis* infected cells, or whole (i.e., non-fragmented) *E. canis* or *E. chaffeensis* proteins, including mixtures of whole proteins, natural whole proteins or whole recombinant proteins are impure reagents, which are of limited usefulness in sero-diagnosis due to sensitivity and specificity issues. For instance, Example 1 of the 09/765,739 application demonstrates that assays that use SEQ ID NOs:1 and 2 were more sensitive and specific than assays that use partially purified *E. canis* antigens. See e.g., paragraph spanning page 20 and 21 of the 09/765,739 application. The partially purified *E. canis* antigens were obtained from *E. canis* organisms grown in tissue culture and partially purified by differential centrifugation and column chromatography. These partially purified *E. canis*' antigens were therefore, mixtures of whole proteins.

3. Assays for detecting anti-*Ehrlichia* antibodies or fragments as described by Waner, Cadman, Rikihisa, and others are severely limited in usefulness because of sensitivity and specificity issues directly related to the impure nature of the *Ehrlichia* antigen used in these tests. See e.g., page 2, line 21 through page 3, line 2 of the 09/765,739 specification (emphasis added). The instant inventions provide highly purified reagents for the detection *Ehrlichia*, that is, polypeptides of about 18-20 amino acids. The use of SEQ ID NOs:1-7 instead of the impure reagents described above, to for

example, detect *Ehrlichia* provide distinct advantages such as greater sensitivity and specificity in sero-diagnostic assays.

4. Waner teaches that the disclosed ELISA assay for detection of *E. canis* closely correlates to the "gold standard" IFA test. See e.g., p. 243, left col. first full paragraph; page 243, right col., first and second paragraph; page 240, right col., first full paragraph.

5. Cadman teaches that the disclosed dot-blot enzyme linked immunoassay (DBELIA) had a sensitivity of 92% and a specificity of 96% when compared to the IFA. See page 135, paragraph spanning columns. Cadman states that the "study showed the DBELIA to be as sensitive and specific as IFA for the detection of antibodies to *E. canis*." See last paragraph.

6. The polypeptides claimed in the instant application have a sensitivity of 98.5% and a specificity of 100% when compared to western blot analysis. Western blot analysis is more sensitive and more specific than IFA analysis. The IFA had, at one time, been considered the "gold standard" for sero-diagnosis of *Ehrlichia*. However, western analysis is more sensitive and more specific than IFA analysis, which uses whole cells as the antigen resulting in cross-reactivity, specificity, and sensitivity issues. The IFA disclosed in the instant invention had a sensitivity of 88% and a specificity of 0%. The polypeptides of the instant invention perform better than the "gold standard" IFA in this study. The Waner and Cadman assays, however, perform only as well as the IFA. Therefore, one of skill in the art could reasonably conclude that the polypeptides of the instant invention perform better, i.e., provide more sensitive and specific results in sero-diagnostic assays, than the Waner and Cadman assays.

7. Rikihisa teaches the use of recombinant, whole proteins to detect *Ehrlichia* antibodies. Rikihisa does not disclose the sensitivity or specificity of the whole, recombinant proteins in sero-diagnostic assays. However, Ohashi *et al.* (J. Clin. Microbiol. 36:2671 (1998)) (copy attached) teaches that dot blot assays performed with whole *E. canis* rP30 antigen to detect *E. canis* were as sensitive as an IFA assay, specificity was not examined in this study. See page 2678, right column, first full paragraph. The instant invention provides peptides (SEQ ID NOs:1-7) that can provide results that are more sensitive than IFA assays. Therefore, one of skill in the art could reasonably conclude that the peptides of the instant invention are more sensitive and more specific than the antigens reported in Waner, Cadman, and are more sensitive than the antigens reported in Rikihisa.

8. The pure reagents described in the instant inventions have additional advantages as compared to the impure reagents described in Waner, Cadman, and Rikihisa. For example, in experiments performed at IDEXX Laboratories mixtures of SEQ ID NOs:1 and 2 showed no cross-reactivity to *Borrelia burgdorferi*, *A. phagocytophilum*, and uninfected canine serum. See Table 1.

Table 1.

Peptide	# of Samples	Canine Serum	Reactivity
Mixtures of SEQ ID NO:1 and SEQ ID NO:2	157	Uninfected	0/157
	81	<i>E. canis</i>	81/81
	166	<i>Borrelia burgdorferi</i>	0/166
	29	<i>A. phagocytophilum</i>	0/29

9. I hereby certify that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

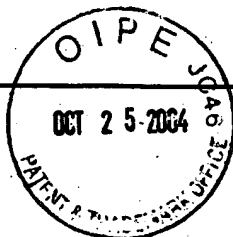
Date: 02/13/2004

By: 
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PROFESSIONAL EXPERIENCE

IDEXX LABORATORIES, WESTBROOK, MAINE

RESEARCH SCIENTIST-II, RAPID ASSAY GROUP, INFECTIOUS DISEASES R&D (2002-PRESENT)

Leading a group responsible for R&D of Point Of Care of Testing devices for companion animals.

HESKA CORPORATION, FORT COLLINS, COLORADO

SENIOR SCIENTIST AND GROUP LEADER, Diagnostic Research (1998-2002)

Management of molecular- and immuno-diagnostic group. Supervised a group of five Scientists (including two Ph.D. Scientists). Designed, developed, and evaluated molecular and immunodiagnostic assays for the diagnosis of parasitic and infectious agents in companion animals. Developed, optimized, and validated immunoassays for cancer markers. Project management: primary person responsible for the management and coordination of projects under aggressive time-lines. Responsible for transfer of assays, documentation and SOPs. Interacted extensively with groups within the company (process and regulatory groups) and outside the organization (external scientific/technical collaborations with both academia and industry).

ACCOMPLISHMENTS

Participated in the research and/or development of the following *Heska* products and reagents:

- *Heska*TM Canine *Ehrlichia* sp. PCR Test (offered as a service in the Heska's Reference Veterinary Diagnostic Laboratory).
- *Heska*TM Feline ImmuCheckTM Assay (Vaccine Titer Assay) (offered as a service in the Heska's Reference Veterinary Diagnostic Laboratory).
- *Heska*TM Equine *Streptococcus equi* PCR Test (offered as a service in the Heska's Reference Veterinary Diagnostic Laboratory).
- *Heska*TM Canine Heartworm Antigen Test (offered as a service in the Heska's Reference Veterinary Diagnostic Laboratory).
- *Heska*TM SoloStepTM CH (USDA Licensed Canine Heartworm Diagnostic POCT).
- *Heska*TM SoloStepTM FH (USDA Licensed Feline Heartworm Diagnostic POCT).
- Seven patents pending.
- Identified several potential diagnostic targets for a fecal diagnostic kit for intestinal helminth infections.

SENIOR SCIENTIST, Nematode Vaccines and Diagnostics (1995-1998)

Management of vaccine antigen discovery and testing group. Supervised one post-doctoral and five associate scientists. Conducted basic research on the biology/ biochemistry/immunology/proteomics of parasitic nematodes to identify potential vaccine antigens. Molecular cloning and expression of recombinant nematode vaccine antigens. Designed and coordinated several animal studies for vaccine efficacy trials (dogs and cats). Antigen discovery research for heartworm diagnostics.

ACCOMPLISHMENTS

- Identified and characterized of over ten potential vaccine candidate nematode antigens against heartworm infections for clinical trials in dogs/cats.
- Five potential recombinant antigens identified by proteomics and cloned for diagnostic evaluation in antibody detection ELISA for feline heartworm infections.
- 13 Patents issued and one pending.

WASHINGTON UNIVERSITY, ST. LOUIS, MISSOURI

RESEARCH ASSISTANT PROFESSOR, Dept. of Medicine and Molecular Microbiology (1995)

RESEARCH INSTRUCTOR, Dept. of Medicine and Molecular Microbiology (1991-1995)

Responsible for immunodiagnostic assay development and vaccine discovery studies in parasitic nematode infections. Field evaluation of diagnostic tests in Egypt. Supervised science technicians.

ACCOMPLISHMENTS

- Developed recombinant antigen-based antibody ELISAs for the diagnosis of human nematode infections. Evaluation of both assays extensively in field studies in Africa.
- Developed a monoclonal antibody-based antigen-based diagnostic assay for human onchocerciasis (river blindness) to detect circulating parasite antigens both in blood and urine.
- Developed a recombinant antigen-based immunoblot assay for diagnosis of Histoplasmosis.
- Identified and tested several candidate recombinant vaccine antigens in animal model of filarial nematode infections.
- One Patent issued and one pending.

NATIONAL/INTERNATIONAL SCIENTIFIC ACTIVITIES

- Invited Participant-WHO: Filariasis Scientific Working Group (UNDP/World Bank/World Health Organization), 1991, 1994. "Protective immunity studies in Onchocerciasis.
- Chairperson: Chaired the scientific session in Filarial Biology. Joint Annual Meeting of the American Society of Tropical Medicine and Hygiene and The American Society of Parasitologists, Atlanta, Georgia. October 31-November 4, 1993.
- Advisor-WHO: Special Program for Research & Training in Tropical Diseases (TDR), World Health Organization, 1993, 1994. Transferred technology from lab research to product development.
- Consultant and collaborating scientist, Epidemiology and Control of Vector Borne Diseases in the Middle East (Egypt-Israel-USA) (USAID/NIH), 1990-1994. Transferred immunodiagnostic assays for lymphatic filariasis from laboratory to field for evaluation studies in Egypt.
- Collaborating Scientist, Participated in protective immunity studies in human filariasis-*International Collaborations in Infectious Disease Research Project (NIH)* to study immunity to filariasis in humans with a longitudinal study of carefully defined populations in a highly endemic region of Egypt, 1994-1995.

POST-DOCTORAL RESEARCH ASSOCIATE (Jewish Hospital of St. Louis at Washington University) (1988-1991)

Identified, characterized and generated monoclonal antibodies to circulating parasitic nematode antigens; Developed antigen detection and recombinant antigen-based antibody assays for human infectious diseases. Participated in a Recombinant DNA Technology Workshop conducted by the *New England Biolabs* and Smith College, Northampton (1991). Supervised a science technician.

CIBA-GEIGY RESEARCH CENTER, BOMBAY, INDIA

SENIOR RESEARCH ASSISTANT (1986-1988)

RESEARCH ASSISTANT (1980-1986)

EDUCATION

Ph.D., Biochemistry - CIBA-GEIGY Research Center, and University of Bombay, India.

MS., Zoology - University of Madras, Madras, India.

HONORS AND AWARDS

National Science Talent Search Scholarship, NCERT, New Delhi, India.

Joshua Gold Medal for best under graduate student.

Joshua Gold Medal and Aaron award for best post graduate student.

National Merit Scholarship, Government of India.

PROFESSIONAL MEMBERSHIPS

American Society of Tropical Medicine and Hygiene.

American Association for the Advancement of Science.

American Society of Parasitologists.

American Society for Microbiologists.

PUBLICATIONS

(SEE ADDENDUM)

Peer-reviewed-64; Invited-7

ADDENDUM

PUBLICATIONS

PEER-REVIEWED

1. Rao RR, Marathe MR, Chandrashekar R, Subrahmanyam D: Ocular filarial infections in *Mastomys natalensis* with *Litomosoides carinii* and *Brugia pahangi*. *Indian J Parasitol* 1983;7:57-60.
2. Reddy AB, Rao UR, Chandrashekar R, Shrivastava R, Subrahmanyam D: Comparative efficacy of some benzimidazoles and amoscanate (Go. 9333) against experimental filarial infections. *Tropenmed Parasitol* (Germany) 1983; 34:259-262.
3. Chandrashekar R, Rao UR, Rajasekariah GR, Subrahmanyam D: Separation of viable microfilariae free of blood cells on Percoll gradients. *J Helminthol* 1984;58:69-70.
4. Chandrashekar R, Rao UR, Subrahmanyam D, Hopper K, Nelson DS, King M: *Brugia pahangi*: Serum-dependent cell-mediated reactions to sheathed and exsheathed microfilariae. *Immunology* 1984;53:411-417.
5. Chandrashekar R, Rao UR, Rajasekariah GR, Subrahmanyam D: Isolation of microfilariae from blood on iso-osmotic Percoll gradients. *Indian J Med Res* 1984;79:497-501.
6. Chandrashekar R, Rao UR, Subrahmanyam D: Effect of diethylcarbamazine on serum dependent cell-mediated reactions to microfilariae in vitro. *Tropenmed Parasitol* (Germany) 1984;35:177-182.
7. Chandrashekar R, Parab PB, Subrahmanyam D: The effect of p-amino-benzoic acid and folic acid on the development of infective larvae of *Brugia malayi* in *Aedes aegypti*. *Acta Trop* (Switzerland) 1984;41:61-67.
8. Reddy AB, Chandrashekar R, Rao UR, Subrahmanyam D: Microfilarial periodicity in *Mastomys natalensis*. *J Helminthol* 1984;58:117-121.
9. Hopper K, Subrahmanyam D, Gregory J, Nelson DS, Rao UR, Chandrashekar R: Mechanisms of ADCC of rat cells to microfilariae: effects of metabolic inhibitors and electron microscopic observations. *Asian Pac J Allergy Immunol* 1984;2:17-21.
10. Chandrashekar R, Rao UR, Subrahmanyam D, Hopper K, Nelson DS, King M: Immune reactions to exsheathed microfilariae of *Litomosoides carinii*. *Indian J Med Res* 1985;81:260-268.
11. Chandrashekar R, Rao UR, Subrahmanyam D: Sharing of antigens among filarial species in antibody-dependent cell-mediated cytotoxicity. *J Biosci* (India) 1985;9:191-196.
12. Chandrashekar R, Rao UR, Parab PB, Subrahmanyam D: *Brugia malayi*: Cell-mediated immune reactions to microfilariae. *Southeast Asian J Trop Med Public Health* 1985;16:15-21.
13. Chandrashekar R, Rao UR, Subrahmanyam D: Serum-dependent cell-mediated immune reactions to *Brugia pahangi* infective larvae. *Parasite Immunol* 1985;7:633-641.
14. Chandrashekar R, Rao UR, Arab PB, Subrahmanyam D: *Brugia malayi*: Rat-cellular interactions with infective larvae mediated by complement. *Exp Parasitol* 1986;62:362-369.
15. Rao UR, Chandrashekar R, Parab PB, Rajasekariah GR, Subrahmanyam D: Lectin-binding characteristics of *Wuchereria bancrofti* microfilariae. *Acta Trop* (Switzerland) 1986;44:35-42.
16. Rao UR, Chandrashekar R, Subrahmanyam D: *Litomosoides carinii*: Characterization of surface carbohydrates of microfilariae and infective larvae. *Tropenmed Parasitol* (Germany) 1986;38:15-18.
17. Rao UR, Chandrashekar R, Subrahmanyam D: Effect of Ivermectin on serum-dependent cellular interactions to *Dipetalonema viteae* microfilariae. *Tropenmed Parasitol* (Germany) 1986;38:123-126.
18. Rao UR, Chandrashekar R, Rajasekariah GR, Subrahmanyam D: Wheatgerm agglutinin binds to infective larvae of *Wuchereria bancrofti* but not to those of *Brugia malayi* and *Brugia pahangi*. *J Parasitol* 1987;73:1256-1258.
19. Rao UR, Chandrashekar R, Subrahmanyam D: Complement activation by eggs and microfilariae of filarial parasites. *Immunol Cell Biol* (Australia) 1987;65:365-370.
20. Rao UR, Chandrashekar R, Subrahmanyam D: Developmental changes in surface carbohydrates of filariae. *Indian J Med Res* 1988;87:9-14.
21. Parab PB, Rajasekariah GR, Chandrashekar R, Alkan SS, Subrahmanyam D: Characterization of a monoclonal antibody specific to the infective larvae of *Brugia malayi*. *Immunology* 1988;64:169-174.
22. Rajasekariah GR, Mukherjee P, Chandrashekar R, Subrahmanyam D: *Brugia pahangi*: Clearance of circulating microfilariae in immunized mice. *Immunol Cell Biol* (Australia) 1988;66:331-336.
23. Rajasekariah GR, Deshpande L, Parab PB, Chandrashekar R, Subrahmanyam D: *Wuchereria bancrofti* larvae in naturally infected *Culex quinquefasciatus*. *Ann Trop Med Parasitol* (Liverpool) 1988;82:637-639.
24. Chandrashekar R, Yates JA, Weil GJ: Use of parasite antigen detection to monitor macrofilaricidal therapy in *Brugia malayi* infected jirds. *J Parasitol* 1990;76:122-124.
25. Chandrashekar R, Rao UR, Subrahmanyam D: IgG response of rats to excretory-secretory products of *Litomosoides carinii*. *Parasitol Res* (Germany) 1990;76:420-423.
26. Chandrashekar R, Rao UR, Subrahmanyam D: Immune response to *Acanthocheilonema viteae* infection in multimammate rat (*Mastomys natalensis*). *Immunol Cell Biol* (Australia) 1990;68:21-26.
27. Weil GJ, Chandrashekar R, Liftis F, McVay CS, Bosshardt SC, Klei TR: Circulating parasite antigen in *Brugia pahangi* infected jirds. *J Parasitol* 1990;76:78-84.
28. Weil GJ, Ogunrinade AF, Chandrashekar R, Kale OO: IgG₄ subclass serology for onchocerciasis. *J Infect Dis* 1990;161:549-554.
29. Chandrashekar R, Rao UR, Subrahmanyam D: Antibody-mediated cytotoxic effects in vitro and in vivo of rat cells on infective larvae of *Brugia malayi*. *Int J Parasitol* 1990; 20:725-730.

30. Chandrashekar R, Ogunrinade AF, Alvarez RM, Kale OO, Weil GJ: Circulating immune-complex associated parasite antigens in human onchocerciasis. *J Infect Dis* 1990;162:1159-1162.
31. Rao UR, Chandrashekar R, Subrahmanyam D: Effect of Ivermectin on filarial infections of *Mastomys natalensis*. *Parasitol Res* (Germany) 1990;76:521-526.
32. Chandrashekar R, Subrahmanyam D, Weil GJ: Effect of CGP 20376 on *Brugia malayi* and parasite antigenemia in jirds. *J Parasitol* 1991;77:479-482.
33. Chandrashekar R, Masood K, Alvarez RM, Ogunrinade AF, Lujan R, Richards FO, Weil GJ: Molecular cloning and characterization of recombinant parasite antigens for immunodiagnosis of onchocerciasis. *J Clin Invest* 1991;88:1460-1466.
34. Li BW, Chandrashekar R, Alvarez RM, Liftis F, Weil GJ: Identification of paramyosin as a potential protective antigen against *Brugia malayi* infection in jirds. *Mol Biochem Parasitol* 1991;49:315-324.
35. Rao UR, Chandrashekar R, Subrahmanyam D: Cell-mediated cytotoxicity of sera from diethylcarbamazine- and Ivermectin-treated rats on microfilariae *in vitro*. *Trop Biomed* 1991;8:1-6.
36. Rajasekariah GR, Parab PB, Chandrashekar R, Deshpande L, Subrahmanyam D: Pattern of *Wuchereria bancrofti* microfilaremia in young adolescent school students in Bassein, an endemic area for lymphatic filariasis. *Ann Trop Med Parasitol* (Liverpool) 1991;85:663-666.
37. Ogunrinade AF, Kale OO, Chandrashekar R, Weil GJ: Field evaluation of IgG₄ serology for the diagnosis of onchocerciasis in children. *Trop Med Parasitol* (Germany) 1992;43:59-61.
38. Weil GJ, Li BW, Liftis F, Chandrashekar R: *Brugia malayi*: Antibody responses to larval antigens in infected and immunized jirds. *Exp Parasitol* 1992;74:315-323.
39. Ogunrinade AF, Chandrashekar R, Weil GJ, Kale OO: Use of a recombinant antigen (OC 3.6) for the serological diagnosis of onchocerciasis in exposed Nigerian children. *J Trop Pediatr* (London) 1992;38:103-105.
40. Li BW, Chandrashekar R, Weil GJ: Vaccination with recombinant filarial paramyosin induces partial immunity to *Brugia malayi* infection in jirds. *J Immunol* 1993;150:1881-1885.
41. Ogunrinade AF, Chandrashekar R, Eberhard ML, Weil GJ: Preliminary evaluation of recombinant *Onchocerca volvulus* antigens for serodiagnosis of onchocerciasis. *J Clin Microbiol* 1993;31:1741-45.
42. Chandrashekar R, Ogunrinade AF, Henry RW, Lustigman S, Weil GJ: *Onchocerca volvulus*: Characterization of monoclonal antibodies to immune complex-associated parasite antigens. *Exp Parasitol* 1993;77:224-234.
43. Chandrashekar R, Curtis KC, Ramzy RM, Liftis F, Li B-W, Weil GJ: Molecular cloning of *Brugia malayi* antigens for diagnosis of lymphatic filariasis. *Mol Biochem Parasitol* 1993;64:261-271.
44. Chandrashekar R, Curtis KC, Weil GJ: Molecular characterization of a circulating parasite antigen in sera from onchocerciasis patients that is immunologically cross-reactive with human keratin. *J Infect Dis* 1995;171:1586-1592.
45. Eberhard ML, Tsang VC, Ottesen EA, Chandrashekar R, Weil GJ, Dickerson JW, Walker EM: Comparison of parasitologic and immunologic responses in primates experimentally infected with *Onchocerca volvulus*. *Exp Parasitol* 1995;80:454-462.
46. Mehta K, Chandrashekar R: Transglutaminase activity in the microfilarial sheath. *Parasitology Today* 1995;11:254.
47. Chandrashekar R, Van Swinderen B, Taylor H, Weil J: Effect of ivermectin prophylaxis on antibody responses to *Onchocerca volvulus* recombinant antigens in experimentally infected chimpanzees. *Int J Parasitol* 1995;25:983.
48. Ramzy R, Helmy H, Chandrashekar R, Gad A, Faris R, Weil GJ: Evaluation of a recombinant antigen-based antibody assay for lymphatic filariasis in Egypt. *Ann Trop Med Hyg* 1995;89:443-446.
49. Singh RN, Chandrashekar R, Mehta K: Purification and partial characterization of transglutaminase from a dog filarial parasite, *Dirofilaria immitis*. *Int J Biochem Cell Biol* 1995;27:1285.
50. Chandrashekar R, Curtis KC, Li BW, Weil G: Molecular characterization of a *Brugia malayi* intermediate filament protein which is a major excretory-secretory product of adult worms. *Mol Biochem Parasitol* 1995;73:231.
51. Mehta K, Chandrashekar R, Rao UR: Transglutaminase-catalyzed incorporation of a host protein (p68) on the surface of *Brugia malayi* microfilariae. *Mol Biochem Parasitol* 1996;76:105.
52. Weil G, Ramzy RM, Chandrashekar R, Gad AM, Lowrie RC, Jr, Faris R: Parasite antigenemia without microfilaremia in bancroftian filariasis. *Am J Trop Med Hyg* 1996;55:333.
53. Chandrashekar R, Ogunrinade AF, Weil GJ: Use of recombinant *Onchocerca volvulus* antigens for diagnosis and surveillance. *Tropical Medicine and International Health* 1996;1:575.
54. Chandrashekar R, Curtis KC, Rawot BW, Kobayashi G, Weil GJ: Molecular cloning and characterization of a recombinant *Histoplasma capsulatum* antigen for diagnosis of human histoplasmosis. *J Clin Microbiol* 1997;35, 1071-1076.
55. Klimowski L, Chandrashekar R, Tripp CA: Cloning, expression and enzymatic activity of a thioredoxin peroxidase from *Dirofilaria immitis*. *Mol Biochem Parasitol* 1997;90:297.
56. Chandrashekar R, Tsuji T, Morales T, Ozols V, Mehta K: An ERp60-like protein from the filarial parasite *Dirofilaria immitis* has both transglutaminase and protein disulfide isomerase activity. *Proc Natl Acad Sci USA* 1998;95:531.
57. Tsuji N, Morales T, Ozols V, Carmody A, Chandrashekar R: Molecular characterization of a calcium-binding protein from the filarial parasite *Dirofilaria immitis*. *Mol Biochem Parasitol* 1998;97:69.

58. Chandrashekar R, Kurtis, KC, Lu, W, Weil GJ. Molecular cloning of an enzymatically active thioredoxin peroxidase from *Onchocerca volvulus*. *Mol Biochem Parasitol* 1998; 93:309.
59. Tsuji N, Morales T, Carmody A, Ozols V, Chandrashekar R. Identification of an asparagine amidohydrolase from the filarial parasite *Dirofilaria immitis*. *Int J Parasitol* 1999; 29:1451.
60. Helmy H, Weil GJ, Faris R, Gad AM, Chandrashekar R, Ashour A, Ramzy RMR. Human antibody responses to *Wuchereria bancrofti* infective larvae. *Parasite Immunol* 2000; 22:89.
61. Chandrashekar R, Tsuji N, Morales T, Carmody A, Ozols V, Welton J, Tang L. Cloning and characterization of a l-Cys peroxiredoxin from the filarial parasite *Dirofilaria immitis*. *Parasitol Res* 2000; 86:200.
62. Tsuji N, Morales T, Ozols V, Carmody A, Chandrashekar R. Cloning and preliminary Characterization of a novel cuticular antigens from the filarial parasite *Dirofilaria immitis*. *Parasitol Int* 2000; 49:321.
63. Ogunrinade AF, Awolola SO, Rotimi O, Chandrashekar R. Longitudinal studies of skin microfilaria and antibody conversion rates in children living in an endemic focus of onchocerciasis in Nigeria. *J Trop pediatr* 2000; 46:348.
64. Chandrashekar R, Devarajan E, Mehta K. *Dirofilaria immitis*: further characterization of the transglutaminase enzyme and its role in larval molting. *Parasitology Res* 2002; 88:185.

INVITED PUBLICATIONS

1. Mehta K, Chandrashekar R, Rao UR: Recent developments in antifilarial agents. *Current Opinion in Therapeutic Patents* (UK) 1992;2:641.
2. Chandrashekar R. Recent advances in the diagnosis of filarial infections. *Ind J Exp Biology*. 1997; 35:18.
3. Frank, GR, Sabin, EA, Chandrashekar, R. Heartworm Vaccine and Immunology Research. In: *Recent Advances in Heartworm Disease: Symposium '98*. Ed. Seward RL. American Heartworm Society, Batavia, IL. 1998, pp 247-256.
4. Chandrashekar R, Mehta K. Transglutaminase-catalyzed reactions in the growth, maturation, and development of parasitic nematodes. *Parasitology Today*. 2000; 11-17.
5. Chandrashekar R. Molecular Mechanisms of immune evasion by *Dirofilaria immitis*. Heartworm Vaccine and Immunology Research. In: *Recent Advances in Heartworm Disease: Symposium '01*. Ed. Seward RL. American Heartworm Society, Batavia, IL. 2001 (In Press).
6. Chandrashekar R. Novel Parasite Transglutaminase-Potential Drug Target. *Ind J Exp Biol*, 2002 (In Press).
7. Mehta K, Rao UR, Chandrashekar R. Transglutaminases of the lower organisms: Across the link between life and death. *Minerva Biotechnologica*, 2002 (In Press).

Cloning and Characterization of Multigenes Encoding the Immunodominant 30-Kilodalton Major Outer Membrane Proteins of *Ehrlichia canis* and Application of the Recombinant Protein for Serodiagnosis

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A 30-kDa major outer membrane protein of *Ehrlichia canis*, the agent of canine ehrlichiosis, is the major antigen recognized by both naturally and experimentally infected dog sera. The protein cross-reacts with a serum against a recombinant 28-kDa protein (rP28), one of the outer membrane proteins of a gene (*omp-1*) family of *Ehrlichia chaffeensis*. Two DNA fragments of *E. canis* were amplified by PCR with two primer pairs based on the sequences of *E. chaffeensis omp-1* genes, cloned, and sequenced. Each fragment contained a partial 30-kDa protein gene of *E. canis*. Genomic Southern blot analysis with the partial gene probes revealed the presence of multiple copies of these genes in the *E. canis* genome. Three copies of the entire gene (*p30*, *p30-1*, and *p30a*) were cloned and sequenced from the *E. canis* genomic DNA. The open reading frames of the two copies (*p30* and *p30-1*) were tandemly arranged with an intergenic space. The three copies were similar but not identical and contained a semivariable region and three hypervariable regions in the protein molecules. The following genes homologous to three *E. canis* 30-kDa protein genes and the *E. chaffeensis omp-1* family were identified in the closely related rickettsiae: *wsp* from *Wolbachia* sp., *p44* from the agent of human granulocytic ehrlichiosis, *msh-2* and *msh-4* from *Anaplasma marginale*, and *map-1* from *Cowdria ruminantium*. Phylogenetic analysis among the three *E. canis* 30-kDa proteins and the major surface proteins of the rickettsiae revealed that these proteins are divided into four clusters and the two *E. canis* 30-kDa proteins are closely related but that the third 30-kDa protein is not. The *p30* gene was expressed as a fusion protein, and the antibody to the recombinant protein (rP30) was raised in a mouse. The antibody reacted with rP30 and a 30-kDa protein of purified *E. canis*. Twenty-nine indirect fluorescent antibody (IFA)-positive dog plasma specimens strongly recognized the rP30 of *E. canis*. To evaluate whether the rP30 is a suitable antigen for serodiagnosis of canine ehrlichiosis, the immunoreactions between rP30 and the whole purified *E. canis* antigen were compared in the dot immunoblot assay. Dot reactions of both antigens with IFA-positive dog plasma specimens were clearly distinguishable by the naked eye from those with IFA-negative plasma specimens. By densitometry with a total of 42 IFA-positive and -negative plasma specimens, both antigens produced results similar in sensitivity and specificity. These findings suggest that the rP30 antigen provides a simple, consistent, and rapid serodiagnosis for canine ehrlichiosis. Cloning of multigenes encoding the 30-kDa major outer membrane proteins of *E. canis* will greatly facilitate understanding pathogenesis and immunologic study of canine ehrlichiosis and provide a useful tool for phylogenetic analysis.

Canine ehrlichiosis is caused by *Ehrlichia canis*, an obligatory intracellular bacterium. It was described originally in Algeria in 1935 (7), and it has now been reported throughout the world and at higher frequency in tropical and subtropical regions (13, 15, 32). Canine ehrlichiosis is characterized by fever, depression, anorexia, and weight loss in the acute phase, with laboratory findings of thrombocytopenia and hypergammaglobulinemia (3, 9). A subclinical phase follows the acute phase (5, 12, 28). In the chronic phase, in addition to the clinical signs and laboratory findings of the acute phase, hemorrhages, epistaxis, edema, and hypotensive shock may occur, which are often exacerbated by superinfection with other organisms (3, 9, 16).

Among several protein antigens of *E. canis*, the proteins in the 30-kDa range were shown to be dominant antigens and

consistently recognized by sera from both experimentally and naturally infected dogs in Western blot analysis (14, 25, 26). The proteins of *E. canis* immunologically cross-react with *Ehrlichia chaffeensis* major antigens in the 30-kDa range (25). These *E. canis* and *E. chaffeensis* proteins were found to be major outer membrane proteins (OMPs) (22). Analysis of a 28-kDa major OMP (P28) gene of *E. chaffeensis*, one of the 30-kDa-range antigens, and its gene copies revealed that these proteins are encoded by a polymorphic multigene family (22). The rabbit serum against a recombinant *E. chaffeensis* P28 protein cross-reacted with the 30-kDa protein of *E. canis* (22).

Dot immunoblot assaying has been developed for serodiagnosis of several infectious agents (4, 10, 11, 30). The advantages of the assay are that an expensive instrument is not required and the interpretation of the results is easy, since positive and negative reactions can be distinguished by the naked eye. However, to be used as the antigen, purification of the organism from infected cells is essential, since *E. canis* is an obligate intracellular bacterium. Purification of *E. canis* is time-consuming and expensive, and serial passages of *E. canis*

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in the cell culture may produce batch-to-batch variation. Although, no genes of *E. canis* other than the 16S rRNA gene have thus far been identified, preparation of a recombinant major antigen is expected to greatly improve the serodiagnosis of *E. canis* infection.

In this study, three genes encoding the 30-kDa OMPs from the *E. canis* genome were identified. All were found to be homologous and phylogenetically characterized. A recombinant protein of *E. canis* which was expressed as a fusion protein was found to be highly antigenic. The dot immunoblot assay was developed with the recombinant *E. canis* protein.

MATERIALS AND METHODS

Organisms and purification. *E. canis* Oklahoma and *E. chaffeensis* Arkansas were cultivated in the DH82 dog macrophage cell line and purified by Percoll density gradient centrifugation (22) or Sephacryl S-1000 column chromatography (26).

PCR, cloning, and expression. The sequences of two forward primers, FECH1 and FECH2, were 5'-CGGGATCCGAATTCGG(A/T/G/C)AT(A/T/C)AA(T/C)GG(A/T/G/C)AA(T/C)TT(T/C)TA-3' and 5'-CGGGATCCGAATTCCTA(T/C)AT(A/T)AG(T/C)GG(A/T/G/C)AA(A/G)TA(T/C)ATG-3', corresponding to amino acid positions 6 to 12 and positions 12 to 18, respectively, of the mature 28-kDa protein (P28) of *E. chaffeensis* (22). These primers have a 14-bp sequence (underlined) at the 5' end to create an *EcoRI* site and a *BamHI* site for insertion into an expression vector. The sequence of a reverse primer, REC1, was 5'-ACCTAATTCCTTGGTAAG-3', complementary to the DNA sequence corresponding to amino acid positions 185 to 191 of the mature P28 of *E. chaffeensis* (22).

Genomic DNA of *E. canis* was isolated from Percoll gradient-purified organisms as described elsewhere (22). PCR amplification was performed by using a Perkin-Elmer Cetus DNA Thermal Cycler (model 480). The 0.6-kb products were amplified with both primer pairs, FECH1-REC1 and FECH2-REC1, and were cloned in the pCRII vector of a TA cloning kit (Invitrogen Co., San Diego, Calif.). The clones obtained by FECH1-REC1 and FECH2-REC1 were designated pCRIIp30 and pCRIIp30a, respectively. Both strands of the insert DNA were sequenced by a dideoxy termination method with an Applied Biosystems 373 DNA sequencer.

For expression, the 0.6-kb fragment was excised from the clone pCRIIp30 by *EcoRI* digestion, ligated into *EcoRI* site of a pET29a expression vector, and amplified in *Escherichia coli* BL21(DE3)pLys (Novagen, Inc., Madison, Wis.). The clone (designated pET29p30) produced a fusion protein with 35-amino-acid and 21-amino-acid sequences carried from the vector at the N and C termini, respectively.

For purification of a recombinant P30 fusion protein (rP30), the cultivated clone was harvested at 4 h after induction with β -D-thiogalactopyranoside. The recombinant protein in the clone pET29p30 was enriched in the pellet by three cycles of centrifugation of the lysate after disruption of the transformant by freezing-thawing and sonication. The final pellet was used as a partially purified rP30 antigen. Affinity-purified rP30 protein was obtained by chromatography with His-Bind Resin (Novagen, Inc.). Briefly, after preparation of the partially purified rP30 antigen, the insoluble protein was extracted with binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9]), including 6 M urea. After being applied to a Ni²⁺-conjugated column, the recombinant protein was eluted with elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9]) containing 6 M urea. The refolding of the purified protein was achieved by sequential dialysis in 20 mM Tris-HCl (pH 7.9) containing 4 and 2 M urea and finally in 20 mM Tris-HCl buffer only and stored at -80°C until use.

Southern blot analysis. Genomic DNA extracted from the Percoll-purified *E. canis* (200 ng each) was digested with restriction enzymes, electrophoresed, and transferred to a Hybond-N⁺ nylon membrane (Amersham, Arlington Heights, Ill.) by a standard method (27). The 0.6-kb DNA inserts containing partial p30 and p30a genes, cloned in pCRIIp30 and pCRIIp30a, respectively, were separately labeled with [α -³²P]dATP by the random primer method with a kit (Amersham), and each labeled fragment was used for Southern blot analysis as a DNA probe. Hybridization was performed at 60°C in Rapid Hybridization buffer (Amersham) for 20 h. The nylon sheet was washed in 0.1× SSC (1× SSC containing 0.15 M sodium chloride and 0.015 M sodium citrate) with 1% sodium dodecyl sulfate (SDS) at 55°C, and the hybridized probes were exposed to Hypermfilm (Amersham) at -80°C.

Cloning and sequencing of 30-kDa protein gene copies from the *E. canis* genomic DNA. The *HindIII* DNA fragment, which was detected by genomic Southern blot analysis as described above, was inserted into pBluescript II KS(+) vectors, and the recombinant plasmids were introduced into *E. coli* DH5 α . By using the colony hybridization method (27), two positive clones which contained chloramphenicol DNA fragments of 3.6 and 7.3 kb were isolated with the ³²P-labeled inserts of pCRIIp30 and pCRIIp30a as probes, respectively. DNA sequencing was performed with suitable synthetic primers by the dideoxy termination method described above.

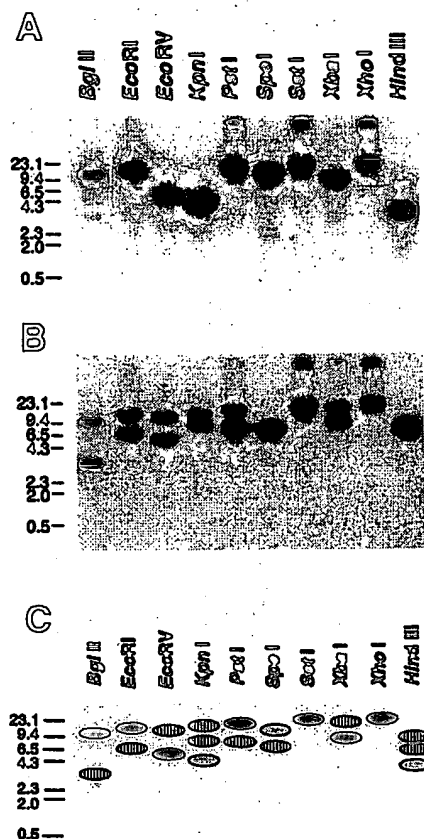


FIG. 1. Genomic Southern blot analysis of *E. canis* DNA with the partial p30 gene probe (A) and with the partial p30a gene probe (B) and schematic representation of the blotting patterns (C). Numbers indicate molecular sizes in kilobases. Filled dots, bands hybridized with both p30 and p30a probes; striped dots, bands hybridized with p30a probe alone; lightly shaded dots, bands hybridized with p30 probe alone.

Sequence analysis. DNA and amino acid sequences were analyzed with the programs DNASIS (Hitachi Software Engineering America, Ltd., San Bruno, Calif.) and DNASTAR (DNASTAR Inc., Madison, Wis.). The amino acid sequences were aligned by using the CLUSTAL method in the DNASTAR program. Phylogenetic analysis was performed by using the PHYLIP software package (version 3.5) (8). An evolutionary distance matrix, generated by using the Kimura formula in the program PROTDIST in the package, was used for construction of a phylogenetic tree by using the unweighted pair-group method of analysis (8). The data were examined by using parsimony analysis (PROTPARS in the PHYLIP). A bootstrap analysis was carried out to investigate the stability of randomly generated trees by using SEQBOOT and CONSENSE in the same package.

Dog plasma and mouse serum. Totals of 34 and 8 dog blood samples with heparin or EDTA were obtained from the Southwest Veterinary Diagnostic Center (Phoenix, Ariz.) and at the Ohio State University Veterinary Teaching Hospital, respectively. All blood specimens collected were centrifuged at 250 × g for 5 min, and the plasma samples were used for this study. For Western blot analysis, these plasma samples were preabsorbed three times with pET29a-transformed *E. coli* at 4°C overnight prior to use. For preparation of the mouse anti-rP30 serum, a male mouse (BALB/c) was intraperitoneally immunized a total of four times at 10-day intervals, once with an equal mixture of the affinity-purified rP30 (30 µg of protein) and Freund's complete adjuvant (Sigma) and three times with an equal mixture of the protein (30 µg) and Freund's incomplete adjuvant. The mouse was sacrificed 7 days after final immunization, and the serum was prepared from blood collected from the heart.

IFA and Western blot analysis. Indirect fluorescent antibody assays, (IFA) and Western blot analysis were performed by a procedure described elsewhere (25). Fluorescein isothiocyanate-conjugated goat anti-dog immunoglobulin G (IgG; Organon Teknica Co., Durham, N.C.) and peroxidase-conjugated affinity-purified anti-dog IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) were used at dilutions of 1:200 for IFA and 1:2,000 for Western blot analysis, respectively, as secondary antibodies.

	SV	HV1	
P30	MNCKRPFPIAS ALISLMSFLP SVSPSESIHE DNIN-GN--- -FYISAKYMP SASHFVGVSF KE-----EKN TTTGVFGLKQ DWDGATIKDA SSSHTIDPST IPSISNYSFK	100	
P30-1	...KIL.TT...YSI...I...DT.QD G.MG-----G...V...V...S...A...SK.S.V...H...SP.LGN KHAD-----T.VP...R	93	
P30a	...KY.KT.TVT...VL.T...TH FIP.YSPARA ST.H---G...G...T...I...A...QS P.KVLV...D...RLSHNI.NNN DTAKS-----LKVQ...93		
P28	...Y.KV...T...I.S...G...DPA-G SG...G...G...A...R...V...N...SA.SNS PND-----V.TV...93		
OMP-1F	...K...TT T.V...GI...DAVQN...VG---G...G...V...V...Q---R...S...SKN PEN-----T.NVP...94		
OMP-1E	...K...TT V...GI...DPVQD...S---G...G...M...A...P.VALY...E...ISSSS HNDN-----H.NNKG...93		
OMP-1D	...EK...TT TL...GI.L.DPVQD...S---G...G...A...R...V...IE...RCV.SRT TL.D-----T.VP...94		
OMP-1C	...K...TT ALP...GILL...FVQD...SVS---G...G...A...P.VALY...N...VSASS HADA-----D.NNKG...93		
OMP-1B	...Y.KI.VS...I...YQ.ADPVTS NDTGINDSRE G...V.N...I...RK.A E.APINGNTS I.KK...K...--GD.AQ.A N.NRTDPALE	98	
MAP-1	...KI...T...T...V...G...DV.Q E.NPV.S---V...T...KM.I...DSR D.KA...K...VKTPSG NTNS-----TEKD...95		
OMP-1A			
	HV2		
P30	YENPFPLGFA GAIGYSMGGP RVEFVSSEI FDKVQNGNSY KNDAR--KYC ALSRHTGMP Q-AGHQ--- --NKPVFLGN EGLLDISLMI NACYDITIDS MPFSPYICAG	201	
P30-1	...I...I...A...SPHIN...Q...--R...H...SAAM E...--D...--IN.K V.V...191		
P30a	...K...R...I...NS...I.L...H...T.P.N.L.S---HSGHICS DGNSGDMYTA KTD...L...V.F.L...TEK...201		
P28	...I.L...H...T...N...E...--R...H...NSAA DMSSAS---N...F.L...VVGEG I...193		
OMP-1F	...V.L.N...I.L.M...T...N...Y...TH--NSQG KLSNAG---D...L...VISEG I...194		
OMP-1E	...L.S...D...I.L...T...N...E...--R...Q...QDN SGIPKT---S.Y.L.S...F.L...INE...I.L...192		
OMP-1D	...L.S...D...I.L...T...N...E...--R...Y...HL.TET ID.AG---SAS...I...K.F.L...VISEG I...196		
OMP-1C	...I.L...T...GN...--R...D...KAS STRATA---SHY.L...L...VISEG I...192		
OMP-1B	PQ...LIS.S...S...A.D...I.L.AA.QK...A.PD.ND T.SGDY...F G...EDALA---DK.Y.V...ITFM...V T...AEG V.I.A...196		
MAP-1	...V...N...I...T...R.P.GN...--M...--DTAS SSTAGA---TTS.MV...N.T...L...ML.G...V...V...193		
OMP-1A			
	HV3		
P30	IGSDLVSMFE TTNPKISYQG KLGVSYSISP EASVFGGWF HRVIGNEFKD IPA---ITPA GATEINGTQF -TTVTLNICH PGLELOGRFTF	288	
P30-1	...T...I...A.S...I...N...T...I...I...R...V.S NS.T.S.P...A...V...N...278		
P30a	...T...I...QN...LN.T.NS RV...A...K...G...T...LL.D SHIKVQGS--A...DV...I.S...F...287		
P28	...T...I...A...L...I...K...R...T...I.T S.LAGKQNY PAI.I.DV...I...V...281		
OMP-1F	V.T...I...AI...L...N...I...K...R...T...MI.S TS.LTON-H...I...SV...V...N...280		
OMP-1E	V.T...I...A...L...N...I...K...R...T...LKAF VTSS--A.PD LAI...SV...I...N...278		
OMP-1D	V.T...I...AI...L...P...I...K...R...T...MI.S ESALAGKQNY PAI...DVPY...I...N...QL...286		
OMP-1C	V.T...I...AI...L...N...I...K...A...R...ST--LKAF ATPSSAA.PD LA...SV...V...N...280		
OMP-1B	V.A...INV.K DP.L.F...I...I...P.T...V.A.I...YV...G...N.NK...VITPVVLE...PQTTT...AL...IDTGY...G.V.V...283		
MAP-1	...T...VIN.A...L...I...N...I...I...ATSKVP.SS NASSAVSPG PASAI.DV...I...I...V...284		
OMP-1A	...EF.D ALHV.FA...I...I...QLPT KVN.LD.YY...Q...Q.N LNVNHVY.LK ESPKVTSS--AVA...D.AY...G.V.I...81		

FIG. 2. Amino acid sequence alignment of P30, P30-1, and P30a of *E. canis*, seven members of *E. chaffeensis* omp-1 multigene family (P28 and OMP-1A to OMP-1F), and MAP-1 of *C. ruminantium* (Senegal strain). The sequences of the *E. chaffeensis* omp-1 gene family and MAP-1 are from the reports of Ohashi et al. (22) and Van Vliet et al. (31), respectively. Aligned positions of identical amino acids with P30 of *E. canis* are indicated by dots. Gaps (indicated by dashes) were introduced for optimal alignment of all proteins. Bars indicate an SV and three HVs (HV1, -2, and -3). The arrowhead indicate the putative cleavage site of the signal peptide.

Dot immunoblot assay. Protein concentrations of purified *E. canis* and recombinant rP30 antigens were determined by a bicinchoninic acid protein assay (Pierce, Rockford, Ill.) with bovine serum albumin as a standard. These antigens in Tris-buffered saline (TBS; 50 mM Tris-HCl [pH 7.4], 150 mM NaCl) were adsorbed onto a nitrocellulose membrane by using a dot blot apparatus (Bio-Rad Laboratories, Richmond, Calif.), blocked for 30 min with TBS containing 2% milk, air dried, and stored at -20°C until use. For immunoassays, the antigen bound to a nitrocellulose strip was incubated with the plasma samples, which were diluted 1:1,000 in TBS containing 2% milk for 1 h at room temperature. After being washed three times with TBS containing 0.05% Tween 20 (T-TBS),

the strip was incubated with peroxidase-conjugated affinity-purified anti-dog IgG (Kirkgaard) at a dilution of 1:2,000 in TBS containing 2% milk. After being washed with T-TBS, the antibody-bound dot was detected by immersing the strip in a developing solution (0.3% 3,3'-diaminobenzidine tetrahydrochloride [Nacalai Tesque, Inc., Kyoto, Japan] and 0.05% hydrogen peroxide in 70 mM sodium acetate [pH 6.2]). The color intensity was analyzed by using background correction in image analysis software (ImageQuant program; Molecular Dynamics, Sunnyvale, Calif.).

GenBank accession number. The DNA sequences of the p30, p30a, and p30-1 genes of *E. canis* have been assigned GenBank accession numbers AF078553, AF078555, and AF078554, respectively.

RESULTS

Cloning and sequencing of three 30-kDa protein gene copies of *E. canis*. Two 0.6-kb DNA fragments containing partial p30

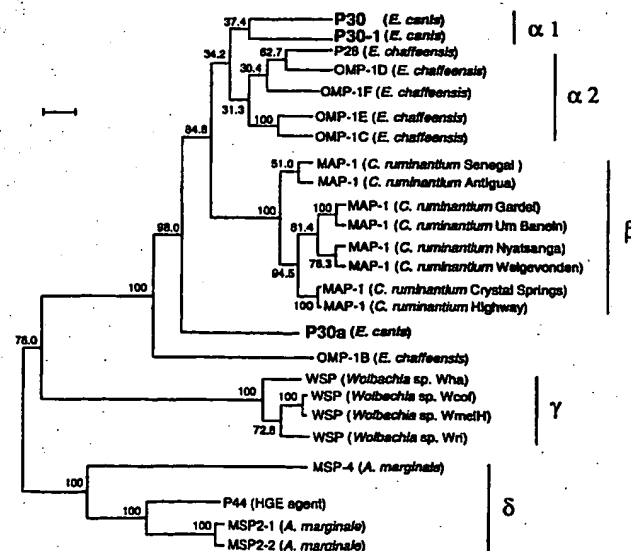


FIG. 3. Phylogenetic classification among P30, P30-1, and P30a of *E. canis* and the major OMPs of the closely related rickettsiae based on amino acid sequence similarities. Evolutionary distance values were determined by the method described by Kimura, and the tree was constructed by the unweighted pair-group method of analysis. Scale bar indicates 10% divergence in amino acid sequences. Bootstrap values from 100 analyses are shown at the branch points of the tree. Bars with symbols indicate representative clusters. The GenBank accession numbers of the major OMP gene sequences of the organisms used in the analysis are as follows: P28 (*E. chaffeensis*), U72291; OMP-1B to OMP-1F (*E. chaffeensis*), AF021338; MAP-1 (*C. ruminantium* Senegal strain), I40882; MAP-1 (*C. ruminantium* Antigua strain), U50830; MAP-1 (*C. ruminantium* Gardel strain), U50832; MAP-1 (*C. ruminantium* Um Bancin strain), U50835; MAP-1 (*C. ruminantium* Nyatsanga strain), U50834; MAP-1 (*C. ruminantium* Welgevonden strain), U49843; MAP-1 (*C. ruminantium* Crystal Springs strain), U50831; MAP-1 (*C. ruminantium* Highway strain), U50833; WSP (*Wolbachia* sp. Wba strain), AF020068; WSP (*Wolbachia* sp. Wcof strain), AF020067; WSP (*Wolbachia* sp. WmelH strain), AF020066; WSP (*Wolbachia* sp. Wri strain), AF020070; MSP-4 (*A. marginale*), Q07408; MSP2-1 (*A. marginale*), U07862; MSP2-2 (*A. marginale*), U36193; and P44 (HGE agent), AF059181.

TABLE 1. Similarities among amino acid sequences of *E. canis* P30, P30-1, and P30a; *E. chaffeensis* omp-1 family (OMP-1B to OMP-1F and P28); *C. ruminantium* MAP-1; *Wolbachia* spp. WSP; HGE agent P44; and *A. marginale* MSP-4, MSP2-1, and MSP2-2

Protein	% Amino acid sequence similarity and evolutionary distance for the following proteins ^a :										
	P30	P30-1	P30a	P28	OMP-1F	OMP-1E	OMP-1D	OMP-1C	OMP-1B	MAP-1 (Senegal)	MAP-1 (Antigua)
P30		80.2	70.8	80.6	80.5	78.6	77.8	77.5	63.2	75.4	76.2
P30-1	0.38628		71.6	79.8	81.7	78.7	78.3	77.3	63.2	74.7	75.6
P30a	0.60811	0.60559		73.9	72.1	73.3	71.2	72.1	58.8	67.2	67.8
P28	0.36288	0.40582	0.50899		85.7	82.3	86.3	81.1	63.6	76.4	77.5
OMP-1F	0.37862	0.36209	0.59907	0.27551		83.4	84.9	83.0	63.2	75.4	75.8
OMP-1E	0.41426	0.42866	0.52142	0.35465	0.32640		81.7	90.1	63.4	76.8	78.1
OMP-1D	0.45193	0.46724	0.61591	0.25793	0.28867	0.36288		81.5	63.2	73.5	74.5
OMP-1C	0.45426	0.48329	0.57469	0.39823	0.34577	0.18285	0.37688		62.4	76.0	77.5
OMP-1B	0.89214	0.87276	0.99793	0.81397	0.83501	0.82982	0.84498	0.89516		62.7	63.2
MAP-1 (Senegal)	0.50490	0.51605	0.76041	0.46987	0.50383	0.46987	0.57453	0.50564	0.92668		93.9
MAP-1 (Antigua)	0.47614	0.50899	0.74635	0.46755	0.52220	0.46096	0.57153	0.48952	0.88842	0.09122	
MAP-1 (Gardel)	0.48606	0.49693	0.72910	0.47185	0.51256	0.46096	0.54403	0.48280	0.89649	0.13499	0.11546
MAP-1 (Crystal Springs)	0.55702	0.53478	0.78883	0.52220	0.56563	0.49693	0.59089	0.53368	0.93601	0.13657	0.14142
MAP-1 (Highway)	0.52891	0.52047	0.76041	0.49443	0.54364	0.46987	0.57594	0.50564	0.93601	0.12383	0.12856
MAP-1 (Nyatsanga)	0.50593	0.49693	0.76544	0.49196	0.53368	0.46755	0.57296	0.48952	0.91855	0.13077	0.11963
MAP-1 (Um Banein)	0.48606	0.49693	0.72910	0.47185	0.51256	0.46096	0.54403	0.48280	0.89649	0.12658	0.11963
MAP-1 (Weigevonden)	0.52629	0.50383	0.74708	0.49877	0.53368	0.47419	0.60290	0.48952	0.92979	0.16080	0.14519
WSP (Wha)	1.57097	1.66864	1.78274	1.59949	1.50435	1.38174	1.61950	1.45510	1.41776	1.58338	1.48404
WSP (Wcof)	1.46262	1.62571	1.62571	1.55195	1.40877	1.29961	1.60271	1.41762	1.33110	1.55897	1.53089
WSP (WmelH)	1.48165	1.64952	1.64952	1.54244	1.39991	1.31514	1.59304	1.43572	1.34750	1.54961	1.49206
WSP (Wri)	1.46435	1.66864	1.70518	1.55687	1.46526	1.27219	1.57654	1.39076	1.32111	1.53292	1.47465
P44	1.77884	1.84928	2.04164	1.56146	1.74020	1.64702	1.64376	1.64702	1.64566	1.57894	1.63909
MSP-4	1.37226	1.39399	1.62744	1.38660	1.45473	1.36494	1.45413	1.47002	1.34294	1.23482	1.31702
MSP2-1	1.50323	1.53992	1.90757	1.40230	1.59474	1.53455	1.40877	1.50435	1.52758	1.53992	1.54847
MSP2-2	1.52476	1.53992	1.87540	1.40230	1.57132	1.53455	1.40877	1.50435	1.55019	1.51796	1.52616

^a Values in the upper right half are percent amino acid sequence similarities; those in the lower left half are evolutionary distances.

and *p30a* genes, amplified by PCR, were cloned and sequenced as described in Materials and Methods. The 0.6-kb DNA, cloned in pCRIIp30a gene were similar but not identical to those of the partial *p30* gene. Genomic Southern blot analysis of *E. canis* digested with several restriction enzymes revealed one and two DNA fragments which could strongly hybridize to the partial *p30* and *p30a* gene probes, respectively (Fig. 1). These restriction enzymes used do not cut within the *p30* and *p30a* gene probes, and, therefore, the result with the *p30a* probe indicates that another gene homologous to the *p30a* is present in the *E. canis* genome. In *Bgl*II, *Eco*RI, and *Pst*I digestion, the *p30* probe hybridized with the upper band of the two *p30a*-hybridized bands. In *Eco*RV and *Xba*I digestion, the *p30* probe hybridized with the lower band of the two *p30a*-hybridized bands. In *Kpn*I, *Spe*I, and *Hind*III digestion, the *p30* probe hybridized with one or two bands that were different from the *p30a*-hybridized bands.

Two DNA fragments of 3.6 and 7.3 kb were cloned by colony hybridization with the probes described above from the *Hind*III-digested genomic DNA of *E. canis*. Sequencing revealed a complete ORF of 864 bp for the *p30* gene in the 3.6-kb fragment and a complete ORF of 861 bp for *p30a* gene in the 7.3-kb DNA fragment. An additional ORF of 921 bp was found in the 3.6-kb DNA. The DNA sequence of the ORF (designated *p30-1*) was also similar but not identical to those of the *p30* and *p30a* genes. There are two potential start codons in the *p30-1* gene sequence. By comparison with the N-terminal amino acid sequences of *p30* and *p30a* genes, we chose a second ATG as a start codon for phylogenetic analysis. The coding region is 834

bp. The *p30-1* and *p30* genes were tandemly arranged with an intergenic space of 355 bp in the 3.6-kb fragment like the *E. chaffeensis* omp-1 family (22). In addition to the result of the genomic Southern blot analysis, this finding showed that at least four homologous genes (*p30*, *p30-1*, *p30a*, and a gene homologous to *p30a*) exist in the *E. canis* genome, suggesting that these genes of *E. canis* are also encoded by a polymorphic multigene family as is the case with *E. chaffeensis* (22).

Structure of proteins encoded by *E. canis* multigenes. Three complete gene copies (*p30*, *p30-1*, and *p30a*) encode 278- to 288-amino-acid proteins with molecular masses of 30,485 to 31,529 Da. The 25-amino-acid sequence at the N termini of P30, P30-1, and P30a (encoded by *p30*, *p30-1*, and *p30a*, respectively) is predicted to be a signal peptide, as described previously (22). The molecular masses of the mature proteins calculated based on the predicted amino acid sequences are 28,750 Da for *p30*, 27,727 Da for *p30-1*, and 29,132 Da for *p30a*.

The predicted amino acid sequences of *E. canis* P30, P30-1, and P30a showed high similarity with those of members in the *E. chaffeensis* omp-1 gene family (22) and that of major antigen protein 1 (MAP-1) of *Cowdria ruminantium* (31). These organisms are also serologically cross-reactive (6, 17, 18, 19, 20). The alignment of amino acid sequences of these proteins revealed substitutions or deletions of one or several contiguous amino acid residues throughout the molecules (Fig. 2). The significant differences in sequences among the proteins are observed in the regions designated SV (semivariable region) and HV (hypervariable region). Computer analysis for hydropathy revealed that protein molecules predicted for three *E. canis* gene copies contain alternative hydrophilic and hydrophobic motifs which are characteristic of typical transmembrane proteins. HV1 and HV2 were located in the hydrophilic regions (data not shown).

TABLE 1—Continued

% Amino acid sequence similarity and evolutionary distance for the following proteins:													
MAP-1 (Gardel)	MAP-1 (Crystal Springs)	MAP-1 (Highway)	MAP-1 (Nyatsanga)	MAP-1 (Um Bancein)	MAP-1 (Welgevonden)	WSP (Wha)	WSP (Wcof)	WSP (WmelH)	WSP (Wri)	P44	MSP-4	MSP2-1	MSP2-2
76.4	74.5	75.4	75.8	76.4	75.2	44.4	44.6	44.4	44.4	19.5	45.6	27.8	27.4
74.7	73.9	74.3	74.7	74.7	74.5	44.0	45.1	44.8	44.6	20.5	47.6	29.3	29.1
67.6	65.9	66.5	66.7	67.6	67.2	41.5	43.2	42.9	42.5	19.5	43.1	24.2	24.2
75.8	74.5	75.4	75.2	75.8	74.9	44.0	44.8	44.8	44.6	22.5	46.9	29.7	29.5
74.5	73.3	73.9	73.9	74.5	73.9	44.6	45.9	45.9	45.3	21.1	46.2	27.8	27.8
76.2	75.4	76.2	76.0	76.2	75.8	45.7	46.9	46.7	46.9	22.0	47.5	28.2	28.0
74.1	73.1	73.5	73.3	74.1	72.4	43.6	44.2	44.2	44.2	22.0	46.0	29.9	29.7
75.8	74.5	75.4	75.6	75.8	75.6	45.3	46.1	45.9	46.1	22.0	46.6	28.6	28.4
63.6	63.2	63.2	63.2	63.6	62.9	45.5	45.1	44.8	45.5	19.1	45.8	26.9	26.5
91.4	90.7	91.4	91.6	91.6	90.1	44.6	45.1	45.1	45.1	21.8	48.8	28.0	28.0
91.8	90.7	91.4	91.6	91.6	90.3	44.8	45.1	45.3	45.3	21.8	48.0	28.0	28.0
	92.2	92.8	94.9	99.6	93.3	44.6	44.4	44.4	44.4	20.9	46.5	27.6	27.4
0.12928		98.9	93.1	92.4	93.1	43.4	43.4	43.4	43.2	20.0	46.1	26.7	26.7
0.11692	0.01764		93.7	93.1	93.7	43.8	43.8	43.8	43.6	20.2	46.5	27.2	27.2
0.08788	0.11285	0.10076		94.5	95.4	43.8	43.8	43.8	43.8	20.5	46.7	28.0	27.8
0.00693	0.12514	0.11285	0.09570		93.3	44.6	44.4	44.4	44.4	20.9	46.5	27.6	27.4
0.11966	0.11285	0.10076	0.08014	0.11966		44.2	44.0	44.0	44.0	20.2	46.5	27.8	27.6
1.51972	1.73099	1.65953	1.64538	1.51972	1.58048		86.1	86.1	90.3	12.5	42.5	22.9	22.7
1.47157	1.59304	1.53089	1.55897	1.47157	1.52893	0.27243		98.3	90.9	13.6	42.1	24.0	24.0
1.46262	1.58338	1.52153	1.54961	1.46262	1.51972	0.26757	0.03029		90.7	13.6	42.3	23.8	23.8
1.44526	1.64362	1.57654	1.53292	1.44526	1.50279	0.18429	0.17605	0.17691		13.6	43.2	24.0	23.8
1.62813	1.74020	1.71093	1.68253	1.62813	1.71093	2.06354	2.15803	2.14440	2.09032		25.7	45.5	45.2
1.33120	1.35101	1.30992	1.31112	1.33120	1.33120	1.72157	1.96007	1.90199	1.72157	1.20170		35.6	34.9
1.50996	1.57836	1.53304	1.46817	1.50996	1.48884	1.70865	1.79325	1.81891	1.72741	0.83164	1.20880		95.6
1.50996	1.55543	1.51116	1.46817	1.50996	1.48884	1.70865	1.75923	1.78382	1.72741	0.84284	1.23930	0.05064	

Phylogenetic relationship among the three *E. canis* 30-kDa proteins and the major OMPs of the closely related rickettsiae based on amino acid sequence similarities. Recently, several major OMP genes which are closely related to the *E. canis* 30-kDa protein have been cloned from rickettsiae (2, 21–24, 31, 34). The phylogenetic tree consisting of 25 major OMPs of the organisms including P30, P30-1, and P30a of *E. canis* was constructed from the estimated evolutionary distances (Fig. 3). The overall pattern of the tree reflects the result based on 16S rRNA gene sequence analysis of the rickettsiae. The 23 representatives, except for *E. canis* P30a and *E. chaffeensis* OMP-1B, are divided into four groups as follows: *E. canis* and *E. chaffeensis*, group α ; *C. ruminantium*, group β ; *Wolbachia* sp., group γ ; and the agent of human granulocytic ehrlichiosis (HGE) and *Anaplasma marginale*, group δ . Group α formed a subcluster of *E. canis* P30 and P30-1 (group α_1), which was separated from another subcluster composed of five *E. chaffeensis* OMPs (group α_2). The similarities between P30 and P30-1 of *E. canis* in group α_1 , between groups α_1 and α_2 , between groups α_1 and β , between groups α_1 and γ , and between groups α_1 and δ were 80.2%, 77.3 to 80.6%, 73.9 to 76.4%, 44.0 to 45.1%, and 19.5 to 47.6%, respectively (Table 1). On the other hand, *E. canis* P30a and *E. chaffeensis* OMP-1B were far from group α and were located between groups β and γ . The similarities between *E. canis* P30a and group α_1 , between P30a and group α_2 , between P30a and group β , between P30a and group γ , and between P30a and group δ were 70.8 to 71.6%, 71.2 to 73.9%, 65.9 to 67.8%, 41.5 to 43.2%, and 19.5 to 43.1%, respectively.

Expression of the *E. canis* p30 gene. The clone pET29p30 produced a 249-amino-acid fusion protein with a molecular mass of 27,316 Da (Fig. 4A). The recombinant protein (rP30) with minimum *E. coli* contamination detectable was obtained in the pellet by centrifugation of the lysate of the transformant (Fig. 4B [partially purified antigen]). The rP30 protein further

purified by affinity chromatography from this preparation had a single band on SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 4B [affinity-purified antigen]). The immunoreactions of *E. canis* rP30 with a total of 42 clinical dog plasma specimens were examined. The IgG-IFA titers of 29 plasma samples were 1:20 to 1:10,480. The remaining plasma samples were IFA negative (<1:20). Western blot analysis revealed that all IFA-positive plasma samples recognized the partially purified rP30 fusion protein (27 kDa) and a 30-kDa protein of

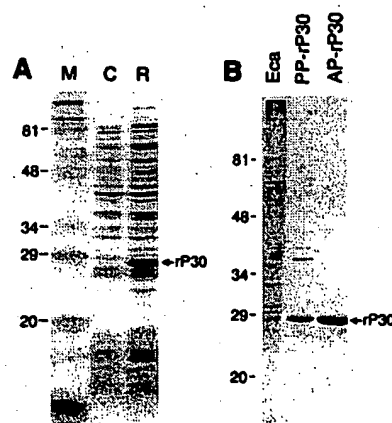


FIG. 4. SDS-PAGE profiles of a recombinant clone expressing P30 of *E. canis* (A) and the purified recombinant protein (B). Gels were stained with Coomassie blue. Lanes: M, molecular size markers; C, pET29-transformed *E. coli* (negative control); R, pET29p30-transformed *E. coli* (recombinant); Eca, purified *E. canis*; PP-rP30, partially purified rP30 fusion protein of *E. canis*; and AP-rP30, affinity-purified rP30 fusion protein. The recombinant rP30 protein is indicated by the arrow. The numbers on the left of each panel indicate molecular masses in kilodaltons.

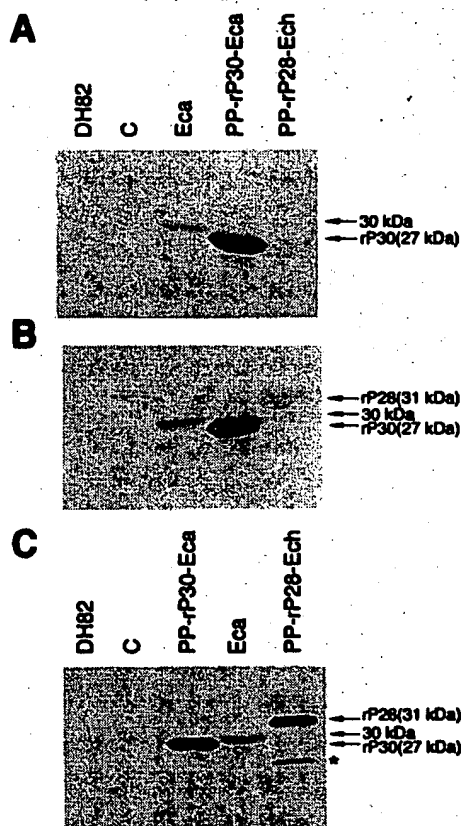


FIG. 5. Western blot analysis with clinical dog plasma with canine ehrlichiosis (A and B) and mouse anti-rP30 serum (C). (A) Dog plasma with a 1:40 IFA titer against *E. canis*; (B) dog plasma with a 1:1,280 IFA titer. Lanes: DH, DH82 dog macrophage cell (negative control); C, a pET29-transformed *E. coli* (negative control); Eca, purified *E. canis* (reactive 30-kDa protein is indicated by arrows in each panel); PP-rP30-Eca, a partially purified rP30 fusion protein (27 kDa) of *E. canis*; and PP-rP28-Ech, a partially purified rP28 fusion protein (31 kDa) of *E. chaffeensis* (22). Another smaller reactive band which may be a degradation product of rP28 of *E. chaffeensis* is indicated by an asterisk.

purified *E. canis* (one of the blots is shown in Fig. 5A), but none of 13 negative plasma samples reacted with any proteins of partially purified rP30 and purified *E. canis* (data not shown). Eight of the 29 positive plasma samples reacted weakly with recombinant P28 fusion protein (rP28 [31 kDa]) of *E. chaffeensis* (22) (one of the blots is shown in Fig. 5B), but the remaining plasma samples did not. A mouse anti-rP30 serum which was prepared by immunization with the affinity-purified antigen reacted with the rP30 antigen, a 30-kDa protein of purified *E. canis*, and an rP28 of *E. chaffeensis* (Fig. 5C). Another smaller band which was observed with *E. chaffeensis* rP28 may be a degradation product of rP28 (asterisk in Fig. 5C), since the plasma sample did not react with *E. coli* proteins. These results showed that rP30 of *E. canis* is highly antigenic and that the antigenic epitope is expressed.

Dot immunoblot assay with the purified whole organism antigen and the recombinant antigen. (i) Optimum amount of antigen per dot. Western blot analysis and dot immunoblot assaying in the preliminary experiments supported the interpretation that there are no significant differences between affinity-purified and the partially purified rP30 in specificity and sensitivity (data not shown). If partially purified recombinant protein is suitable for serodiagnosis, it will be more cost-effective. By dot immunoblot assaying we examined in detail whether

er partially purified rP30 is suitable as an antigen for serodiagnosis.

Nitrocellulose strips having serially diluted purified *E. canis* or partially purified rP30 antigen of *E. canis* were reacted at a 1:1,000 dilution with dog plasma samples with different IFA titers against *E. canis*, and the color intensities of the reaction of each dot were compared (Fig. 6). Dots of 0.01 to 1 μ g of the purified organisms (Fig. 6A) or dots of 0.025 to 1 μ g of rP30 (Fig. 6B) that reacted with positive plasma samples (>1:20 in IFA titer) were clearly distinguishable from those that reacted with negative plasma samples (<1:20) by the naked eye. There was no nonspecific reaction with the negative plasma samples when purified *E. canis* was used as an antigen; however, a weak nonspecific reaction with IFA-negative plasma was observed in dots of 0.25 to 1 μ g of partially purified rP30 antigen. Based on these results, the optimum amounts of antigens per dot were determined to be 1 and 0.5 μ g for antigen proteins of purified *E. canis* and partially purified rP30, respectively. These results show that the partially purified recombinant protein is apparently sufficient as an antigen for serodiagnosis.

(ii) Optimum dilution of antiserum. The immunoreactivities of plasma at dilutions of 1:300, 1:1,000, and 1:3,000 were examined with nitrocellulose strips of the purified *E. canis* an-

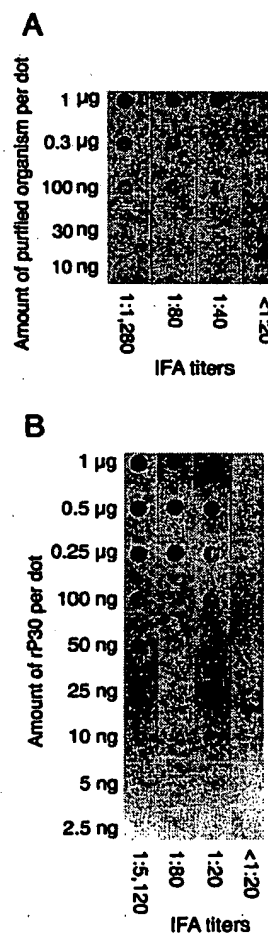


FIG. 6. Optimum amount of antigens for dot blot assaying with purified *E. canis* antigen (A) or partially purified rP30 antigen (B). Purified organism antigen (10 ng to 1 μ g) or rP30 antigen (2.5 ng to 1 μ g) was blotted onto the nitrocellulose sheet, reacted with each plasma at a 1:1,000 dilution as primary antibody, and reacted with secondary antibody (peroxidase-conjugated affinity-purified anti-dog IgG antibody) at a 1:2,000 dilution.

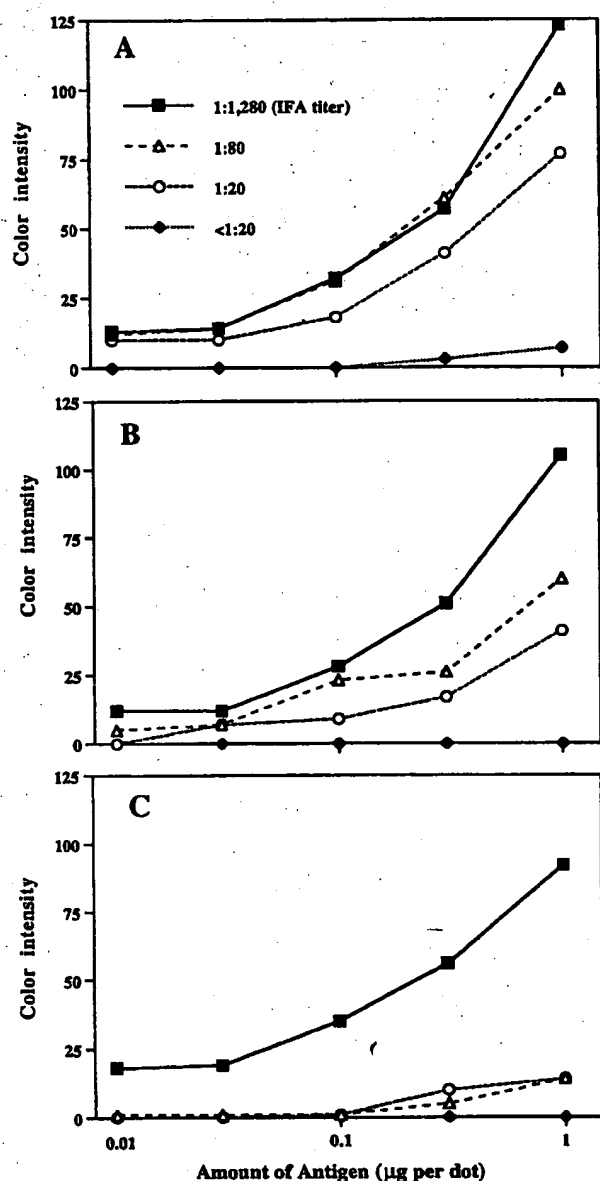


FIG. 7. Optimum plasma dilutions for dot blot assay. Purified *E. canis* antigen was blotted as described in the legend to Fig. 6. The antigens were incubated with plasma at dilutions of 1:300 (A), 1:1,000 (B), and 1:3,000 (C). The plasma samples used were the same as those used for Fig. 6A. The color intensity of each dot was determined by using the image software program (ImageQuaNT).

tigen as shown in Fig. 6A. The color intensity values were plotted in graphs (Fig. 7). At a 1:300 dilution (Fig. 7A), color development occurred in the dots having an antigen greater than 0.3 μg per dot with IFA-negative plasma. At a 1:3,000 dilution (Fig. 7C), color intensities of all plasma samples were low, especially in the case of positive plasma samples with low IFA titers (1:20 and 1:80). At a 1:1,000 dilution (Fig. 7B), positive plasma with even the lowest IFA titer (1:20) was distinguishable from IFA-negative plasma by the naked eye, especially with 1 μg of purified *E. canis* antigen per dot (Fig. 6A). The optimum dilution of plasma for testing was, therefore, 1:1,000.

(iii) Examination of clinical dog plasma with purified *E. canis* and partially purified rP30 antigens. A total of 42 clinical

plasma samples were examined with 1 μg of purified *E. canis* antigen per dot and 0.5 μg of partially purified rP30 antigen per dot (Fig. 8). The plasma samples with higher IFA titers showed a darker reaction with both native and recombinant antigens. The color intensities between plasma with IFA titers of >1:20 and IFA-negative plasma were clearly distinguishable by the naked eye. The correlation between IFA titers and color intensity values by the dot immunoblot assay was examined (Fig. 9). The maximum color intensity values of 13 IFA-negative plasma samples (<1:20) were zero (background) in the purified *E. canis* antigen and 10 in the rP30 antigen. All 29 IFA-positive plasma samples (>1:20) showed color intensity values of greater than 19 in the purified *E. canis* and 18 in the rP30 antigen. The highest color intensity values were 105 in the purified organism and 114 in the rP30 antigen. In both native and recombinant antigens, color intensity values correlated with IFA titers. The correlation coefficients between IFA titers and color intensities of native and recombinant antigens were 0.71 ($P < 0.001$) and 0.68 ($P < 0.001$), respectively. Therefore, it may be possible to estimate an approximate titer of the test serum or plasma by comparing the color densities with those of serially diluted standard serum or plasma.

DISCUSSION

The availability of recombinant immunodominant major surface proteins of *E. canis* will greatly assist in diagnosis and in understanding of the pathogenesis of this intracellular bacterium, such as invasion of host cells, elicitation of the immune response, and mechanisms of the clinical disease. The 30-kDa protein of *E. canis* was shown to be the immunodominant major OMP, which can be recognized by naturally and experimentally infected dog sera (14, 25, 26). Therefore, the 30-kDa protein is the primary recombinant antigen candidate for use in the serodiagnosis of *E. canis* infection. The present study is the first report of molecular characterization of 30-kDa major OMPs of *E. canis*.

Polymorphic multigene families encoding the major OMPs have been identified in *E. chaffeensis*, the HGE agent, and *A. marginale*, which are closely related to *E. canis* based on 16S rRNA gene sequences. Six copies of the *E. chaffeensis* *p28* gene (*omp-1* gene family) are tandemly arranged with intergenic spaces (22), while copies of the HGE agent *p44* gene and the *A. marginale* *msp-2* and *msp-3* genes are distributed widely throughout the genomes (1, 23, 34). In this study, the 30-kDa proteins of *E. canis* were also shown to be encoded by a polymorphic multigene family. The two *E. canis* genes are tandemly arranged with an intergenic space as are members of the *E. chaffeensis* *omp-1* gene family. Although we demonstrated the presence of four gene copies of 30-kDa *E. canis* proteins in the genome, additional gene copies which are tandemly arranged may exist in three genomic *HindIII* DNA fragments which hybridized to *p30* and *p30a* probes. Sequence analysis revealed that the 30-kDa proteins (P30, P30-1, and P30a) of *E. canis* had characteristics of the *E. chaffeensis* OMP-1 family (22) and *C. ruminantium* MAP-1 (31). The *C. ruminantium* MAP-1 has been reported to be cross-reactive to a 27-kDa protein of *E. canis* (19), although it is unknown whether the 27-kDa protein is identical to P30, P30-1, or P30a of *E. canis* in this study. Phylogenetic analysis based on the homologs from the closely related rickettsiae revealed that P30 and P30-1 of *E. canis* are present in the same cluster but that P30a is far from the cluster, suggesting that the multigenes encoding the 30-kDa *E. canis* proteins are widely divergent. Interestingly, in the phylogenetic tree, the 30-kDa *E. canis* proteins, the *E. chaffeensis* OMP-1 family, the HGE agent P44, and *A. mar-*

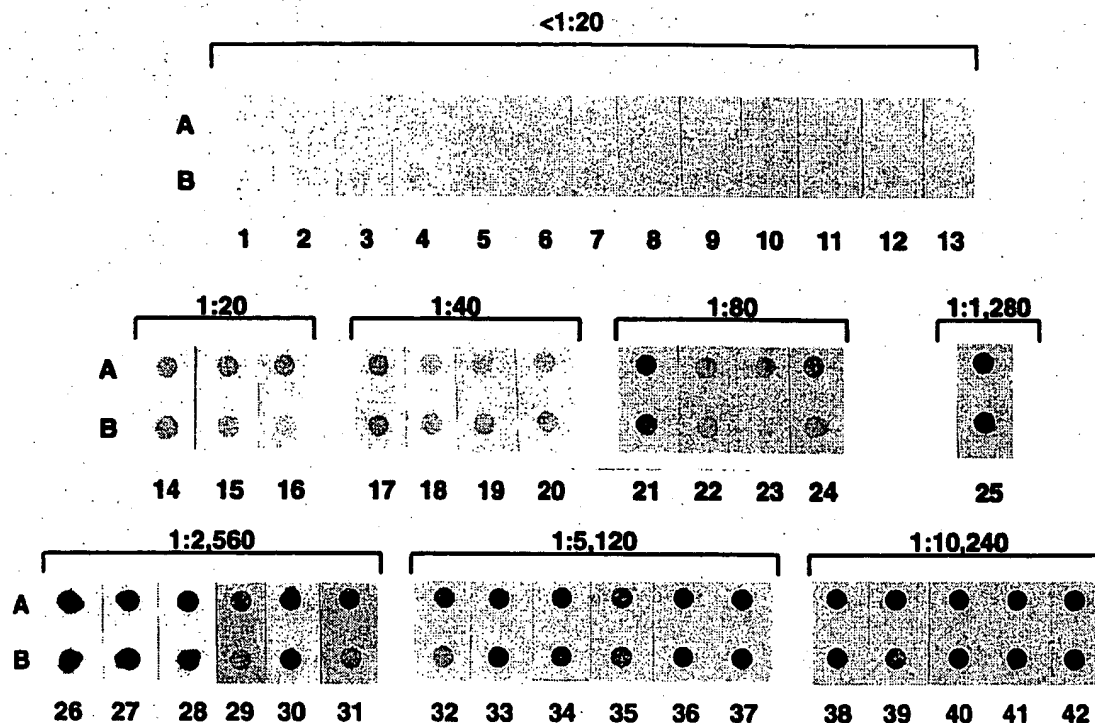


FIG. 8. Reaction profiles of purified *E. canis* antigen (1 μ g) (A) and partially purified rP30 antigens (0.5 μ g) (B) with 42 plasma samples. Plasma identifications are indicated below each dot. Numbers above brackets indicate the IFA titers of the plasma samples.

ginale MSP-2 are encoded by a polymorphic multigene family as described above. However, *C. ruminantium* MAP-1, *Wolbachia* sp. WSP, and *A. marginale* MSP-4 are encoded by a single gene (2, 21–24, 31). The diversities reported among the *C. ruminantium* MAP-1s and among the *Wolbachia* sp. WSPs are strain variation (2, 24, 31).

Molecular analysis of *E. canis* 30-kDa antigens such as ours is important in understanding the antibody responses of animals, because the antigenic diversity may influence the specificity and sensitivity of the serologic assay. Previously, we observed in the Western blot analysis that acute-phase serum (before 30 days postinoculation) from an *E. canis*-infected dog reacted strongly with a 30-kDa protein but weakly with a 31-kDa protein. However, the reactivity of the chronic-phase serum (after 60 days postinoculation) from the same dog was reversed (strong reaction with the 31-kDa protein and weak reaction with the 30-kDa protein) (14). This might be due to differential expression of the multigene encoding the 30-kDa protein of *E. canis* during infection. Although it is unknown whether the genes of P30, P30-1, and P30a were expressed by *E. canis* in tissue culture or in the infected dog, the recombinant P30 protein constructed in this study expressed the antigenic epitope which can react with all IFA-positive dog plasma samples used, suggesting that the antigenic epitope conserved among the 30-kDa protein gene family is expressed. This strongly supports the idea that rP30 is useful as an antigen for serodiagnosis of canine ehrlichiosis.

For serodiagnosis of canine ehrlichiosis, IFA is widely used. However, a fluorescence microscope and trained personnel are required for this test. Furthermore, cell culture of *E. canis* may produce batch-to-batch variation. A consistent and simple assay that can detect specific antibodies without expensive equipment would be an invaluable aid in serodiagnosis. In the dot immunoblot assay, antibody-positive serum can be distin-

guished from antibody-negative serum by the naked eye, and if proper color standards are provided, anyone can easily make the final evaluation. The greatest obstacle for the development of this assay is the production of diagnostic antigens sufficient in purity and amount. If recombinant antigens are available, the antigen preparation would be simpler, more consistent, and economical than purified organism antigen preparation. Previously, a dot blot enzyme-linked immunoassay for detecting antibodies to *E. canis* has been reported (4). However, the crude antigens, freed from host cells by freezing-thawing, were used in that study. Neither recombinant antigens nor the purified antigens (such as organisms purified by Sephacryl S-1000 column chromatography) were used. Additionally, that report contains only one page of description without any data. Therefore, we think our dot immunoblot assay using the recombinant 30-kDa antigen of *E. canis* would greatly enhance serodiagnosis of canine ehrlichiosis.

Recognition of the lowest positive IFA titer (1:20) plasma by a dot immunoblot assay with 1 μ g or less of protein of the whole organism or the recombinant antigen per dot shows that this assay is as sensitive as IFA. Although the specificity of the test, except for cross-reactivity with *E. chaffeensis*, was not analyzed in this study, as with any other serologic test, dot immunoblot assaying probably cannot distinguish among antigenically cross-reactive members of the tribe *Ehrlichieae*. However, the use of recombinant *E. canis* antigen gave greater sensitivity than the use of recombinant *E. chaffeensis* antigen for serodiagnosis of canine ehrlichiosis. Western blot analysis revealed that 8 of 22 IFA-positive plasma samples slightly cross-reacted with recombinant 28-kDa protein of *E. chaffeensis*. This weak cross-reactivity is not a potential problem for clinics, since treatment is the same for all of the ehrlichial agents.

In dot immunoblot assays of 29 IFA-positive plasma sam-

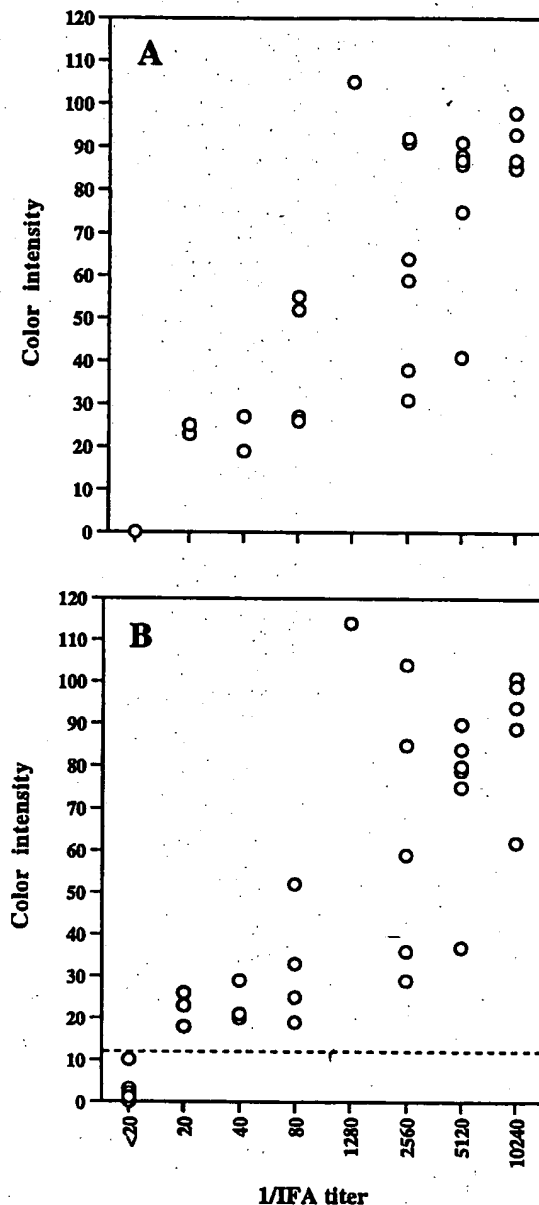


FIG. 9. Correlation between IFA titer (reciprocal dilutions) and color intensity of the dot immunoassay with purified *E. canis* antigen (A) and partially purified rP30 antigen (B). The color intensities of all dots in Fig. 8 were determined and plotted. Each circle represents one plasma specimen ($n = 42$). The correlation coefficients were 0.71 ($P < 0.001$) for graph A and 0.68 ($P < 0.001$) for graph B. The dashed line in graph B represents the cutoff value, which was determined from the highest color intensity in the immunoreaction with 13 negative plasma samples.

ples, 5 had color intensities of the purified organism antigen greater or lesser than those of the recombinant antigens. Additional major immunodominant proteins of *Ehrlichia* spp. are heat shock proteins (HSPs) (29, 33). Consequently, when anti-HSP antibody or antibody against protein antigen other than P30 is present in the plasma, whole organism antigens would give an immunoreaction stronger than that of the recombinant protein. On the contrary, when anti-P30 antibody is dominant in the plasma, the reaction with the recombinant protein would be stronger than that with the whole organism antigen. More

importantly, the recombinant antigen-dot blot assay could clearly detect all of the 29 IFA-positive plasma samples. Furthermore, between native and recombinant antigens, no significant difference was observed in the correlation coefficient between IFA titers and the blot color intensity. Therefore, the rP30 antigen-immunodot blot assay offers advantages over the other serodiagnostic tests in general availability, ease of handling, and accuracy in the serodiagnosis of *E. canis* infection. Additionally, although it was not described in this paper, this *E. canis* recombinant antigen can be applied to enzyme-linked immunosorbent plate assays or other serodiagnostic assays as well.

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REFERENCES

- Alleman, A. R., G. H. Palmer, T. C. McGuire, T. F. McElwain, L. E. Perryman, and A. F. Barbet. 1997. *Anaplasma marginale* major surface protein 3 is encoded by a polymorphic, multigene family. *Infect. Immun.* 65:156-163.
- Braig, H. R., W. Zhou, S. L. Dobson, and S. L. O'Neill. 1998. Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia pipiensis*. *J. Bacteriol.* 180:2373-2378.
- Buhles, W. C., D. L. Huxsoll, and M. Ristic. 1974. Tropical canine pancytopenia: clinical, haematologic, and serologic, and serologic response of dogs to *Ehrlichia canis* infection, tetracycline therapy, and challenge inoculation. *J. Infect. Dis.* 130:358-367.
- Cadman, H. F., P. J. Kelly, L. A. Matthewman, R. Zhou, and P. R. Mason. 1994. Comparison of the dot-blot enzyme linked immunoassay with immunofluorescence for detecting antibodies to *Ehrlichia canis*. *Vet. Rec.* 135:362.
- Codner, E. C., and L. L. Farris-Smith. 1986. Characterization of the sub-clinical phase of ehrlichiosis in dogs. *J. Am. Vet. Med. Assoc.* 189:47-50.
- Dawson, J. E., Y. Rikihisa, S. A. Ewing, and D. B. Fishbein. 1991. Serologic diagnosis of human ehrlichiosis using two *E. canis* isolates. *J. Infect. Dis.* 163:564-567.
- Donatien, A., and F. Lestoquard. 1935. Existence and algerie d'une rickettsia du chien. *Bull. Soc. Pathol. Exot.* 28:418-419.
- Felsenstein, J. 1989. PHYLIP-phylogeny inference package (version 3.3). *Cladistics* 5:164-166.
- Greene, C. E., and J. W. Harvey. 1990. Canine ehrlichiosis, p. 405-414. *In* C. E. Greene (ed.), *Clinical microbiology and infectious diseases of the dog and cat*. The W. B. Saunders Co., Philadelphia, Pa.
- Heberling, R. L., and S. S. Kalter. 1986. Rapid dot immunobinding assay on nitrocellulose for viral antibodies. *J. Clin. Microbiol.* 23:109-113.
- Heberling, R. L., S. S. Kalter, J. S. Smith, and D. G. Hildebrand. 1987. Serodiagnosis of rabies by dot immunobinding assay. *J. Clin. Microbiol.* 25:1262-1264.
- Huxsoll, D. L., P. K. Hildebrandt, R. M. Nims, and J. S. Walker. 1970. Tropical canine pancytopenia. *J. Am. Vet. Med. Assoc.* 157:1627-1632.
- Immelman, A., and C. Button. 1973. *Ehrlichia canis* infection (tropical canine pancytopenia or canine rickettsiosis). *J. S. Afr. Vet. Assoc.* 44:241-245.
- Iqbal, Z., and Y. Rikihisa. 1994. Reisolation of *Ehrlichia canis* from blood and tissues of dogs after doxycycline treatment. *J. Clin. Microbiol.* 32:1644-1649.
- Klopfer, U., and T. A. Nobel. 1972. Canine ehrlichiosis (tropical canine pancytopenia) in Israel. *Refu. Vet.* 29:24-29.
- Kuehn, N. F., and S. D. Gaunt. 1985. Clinical and hematologic findings in canine ehrlichiosis. *J. Am. Vet. Med. Assoc.* 186:355-358.
- Logan, L. L., C. J. Holland, C. A. Mebus, and M. Ristic. 1986. Serological relationship between *Cowdria ruminantium* and certain *Ehrlichia* spp. *Vet. Rec.* 119:458-489.
- Maeda, K., N. Markowitz, R. C. Hawley, M. Ristic, D. Cox, and J. E. McDade. 1987. Human infection with *Ehrlichia canis*, a leukocytic rickettsia. *N. Engl. J. Med.* 316:853-856.
- Mahan, S. M., N. Tebele, D. Mukwede, S. Semu, C. B. Nyathi, L. A. Wassink, P. J. Kelly, T. Peter, and A. F. Barbet. 1993. An immunoblotting diagnostic assay for heat water based on the immunodominant 32-kilodalton protein of *Cowdria ruminantium* detects false positive in field sera. *J. Clin. Microbiol.* 31:2729-2737.
- McDade, J. E. 1990. Ehrlichiosis—a disease of animals and humans. *J. Infect. Dis.* 161:609-617.
- Oberle, S. M., and A. F. Barbet. 1993. Derivation of the complete *msp4* gene sequence of *Anaplasma marginale* without cloning. *Gene* 136:291-294.
- Ohashi, N., N. Zhi, Y. Zhang, and Y. Rikihisa. 1998. Immunodominant major outer membrane proteins of *Ehrlichia chaffeensis* are encoded by a

- polymorphic multigene family. *Infect. Immun.* 66:132-139.
23. Palmer, G. H., G. Eid, A. F. Barbet, T. C. McGuire, and T. F. McElwain. 1994. The immunoprotective *Anaplasma marginale* major surface protein 2 is encoded by a polymorphic multigene family. *Infect. Immun.* 62:3808-3816.
 24. Reddy, R. G., C. R. Sulsona, R. H. Harrison, S. M. Mahan, M. J. Burrige, and A. F. Barbet. 1996. Sequence heterogeneity of the major antigenic protein 1 genes from *Cowdria ruminantium* isolates from different geographical areas. *Clin. Diagn. Lab. Immunol.* 3:417-422.
 25. Rikihisa, Y., S. A. Ewing, and J. C. Fox. 1994. Western immunoblot analysis of *Ehrlichia chaffeensis*, *E. canis* or *E. ewingii* infection of dogs and humans. *J. Clin. Microbiol.* 32:2107-2112.
 26. Rikihisa, Y., S. A. Ewing, J. C. Fox, A. G. Siregar, F. H. Pasaribu, and M. B. Malole. 1992. Analysis of *Ehrlichia canis* and a canine granulocytic *Ehrlichia* infection. *J. Clin. Microbiol.* 30:143-148.
 27. Sambrook, J., T. Maniatis, and E. F. Fritsch. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 28. Stephenson, E. H., E. R. Clothiw, and M. Ristic. 1975. *Ehrlichia canis* infection in a dog in Virginia. *J. Am. Vet. Med. Assoc.* 172:63-64.
 29. Sumner, J. W., K. G. Sims, D. C. Jones, and B. E. Anderson. 1993. *Ehrlichia chaffeensis* expresses an immunoreactive protein homologous to the *Escherichia coli* GroEL protein. *Infect. Immun.* 61:3536-3539.
 30. Urakami, H., S. Yamamoto, T. Tsuruhara, N. Ohashi, and A. Tamura. 1989. Serodiagnosis of scrub typhus with antigens immobilized on nitrocellulose sheet. *J. Clin. Microbiol.* 27:1841-1846.
 31. Van Vliet, A. H. M., F. Jongejan, M. van Kleef, and B. A. M. van der Zeijst. 1994. Molecular cloning, sequence analysis, and expression of the gene encoding the immunodominant 32-kilodalton protein of *Cowdria ruminantium*. *Infect. Immun.* 62:1451-1456.
 32. Wilkins, J. H., R. S. T. Bowden, and G. T. Wilkinson. 1967. A new canine syndrome. *Vet. Rec.* 81:57-58.
 33. Zhang, Y., N. Ohashi, E. H. Lee, A. Tamura, and Y. Rikihisa. 1997. *Ehrlichia sensu lato* groEL operon and antigenic properties of the GroEL homolog. *FEMS Immunol. Med. Microbiol.* 18:39-46.
 34. Zhi, N., N. Ohashi, Y. Rikihisa, H. W. Horowitz, and G. P. Wormser, and K. Hechemy. 1998. Cloning and expression of 44-kilodalton major outer membrane protein gene of the human granulocytic ehrlichiosis agent and application of the recombinant protein to serodiagnosis. *J. Clin. Microbiol.* 36:1666-1673.

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